EDITOR'S VIEWPOINT

Snyder, Suher, Porter, Claycomb, and Gardner (p. 332) report on a phase of their evaluation of laboratory tests for the estimation of caries activity. Comparing the different laboratory tests with each other, they conclude that lactobacillus counts made on tomato juice peptone agar are comparable with those made on synthetic medium when the azide level is 1:20,000 and that there is little to choose between Snyder's test agar and Rogosa's SL (mod) medium. We agree with the authors that the most important phase of this study will be the correlation of the laboratory tests with the progress of caries in the subjects under study.

Adler (p. 344) reports the results of a comparison of caries accumulation (DMF) with occlusal conditions. In general, more caries is associated with malocclusion than with normal occlusion. There is one outstanding exception in retrusion of the maxillary incisors on an hereditary basis. As the author points out, the caries may predispose to malocclusion and occlusion variations may predispose to caries. The term CER (caries, extractions, and restoration) is used rather than DMF to indicate decayed, missing, and filled teeth.

The value of a system of scoring periodontal tissue for epidemiological studies that would serve in a manner similar to the DMF for dental caries is obvious. Russell (p. 350) has developed such a scoring and classification system and has tested its reliability and reproducibility in 200 patients and compared results with other population groups measured independently by the same method. While the system does not include an evaluation of the etiology or the type of periodontal disease, it does appear to measure the degree of destruction resulting from periodontal diseases. Its value would seem to lie in measurement of the potential tooth loss from periodontal diseases, of whatever type, in population groups.

Bowen (p. 360) discusses the possible use of epoxy resins for dental restorations. A method for increasing the adhesion is proposed and the results of tests are given. It is still evident that a completely satisfactory plastic restorative material is not yet available and that the search should include the investigation of different types of plastics as well as efforts to improve those presently used. Investigators working in commercial laboratories, and independently, all have the same goal and should be encouraged.

Schmidt and Burnett (p. 370) measured the reducing activity of hetero- and homofermentative lactobacilli using T.T.C. (2, 3, 5 triphenyltetrazolium chloride) as an indicator. Smaller concentrations on heterofermentative lactobacilli were needed to reduce T.T.C. than those of homofermentative lactobacilli, suggesting a use for T.T.C. in differentiating those two broad groups of lactobacilli. Since maximal reduction was obtained at pH 6, or lower, although flavin enzymes, suggested as those active in T.T.C. reduction, are most active in alkaline media, and since attempts to demonstrate flavin enzyme systems in lactobacilli were negative, other enzyme systems may be active.

Nikiforuk (p. 377) investigated the reducing properties of saliva. Making "painfully exact measurements" upon this "fightfully impure compound" he found no significant differences between salivas from caries-free and caries-active children, found most of the reducing power in the salivary sediment, and found that furacin, but not sodium fluoride or penicillin, would decrease the reducing coefficient. The latter observation is important, for fluorides and penicillin have been shown to reduce caries activity but furacin failed to aid in caries control.

Turner and Anders (p. 385) from data obtained from caries-free and apparently active-caries cases in children suggest that there may be a more important relationship between physiological buffers, on the alkaline side, in the saliva and dental caries

than between the presence of acid and dental caries. Of course, the probable ability of a well-buffered saliva and dental caries suppression has interested those investigating methods for control of dental caries. It has been difficult to demonstrate constant relationship between buffering capacity of saliva and dental caries activity, and, while the problem is far from settled, present studies indicate that titrable alkalinity is more constantly related to the amount of caries than is titrable acidity.

Claycomb, McChesney, and Snyder (p. 391) describe a simple and rapid method for determining the quantity of amylase in saliva.

Rosen, Ragheb, Hoppert, and Hunt (p. 399) were faced with the perplexing result of reduction in acidogenic organisms and caries in caries-susceptible rats consuming feed with penicillin but with only slight reduction of caries and marked reduction in acidogens in those receiving Terramycin. Higher streptococci counts were found in the Terramycin-fed rats but this pattern was also found in caries-resistant (genetically) strains of rats. It is quite possible that lactobacilli are the acid producers under antibiotic-free conditions in susceptibles but that streptococci may be inhibited from performing in caries-resistant animals. Since the use of antibiotics may lead to changes in the morphology and physiology of surviving bacteria, the cocci found after treatment should be subjected to more thorough examination.

Jordan, Fitzgerald, and Faber (p. 404) investigated *Micrococci* isolated from salivas of 46 adults. To those not actively engaged in bacteriology the *Micrococci* include the group formerly known as *Staphylococci* and it is those organisms that are the subject of this study. These organisms were divided into three groups and their biochemical activity investigated. These studies are but a part of the broad investigation of the oral flora that must be continued if we are to understand the normal and pathologic phases of this bacteria-rich region.

Burnett (p. 413) analyzed the effect of sodium and calcium fluoride on the dissolution of calcium, phosphorus, magnesium, and carbon dioxide from dentin. While these elements account for slightly more than half of the weight loss, they were recovered in fairly constant amounts. Total weight loss appears to be a useful indicator of tissue solubility. It should be noted that this study was conducted on dentin to establish a technic, while most interest on solubility after fluoride treatment concerns enamel.

Brudevold, Gardner, and Smith (p. 420) analyzed the fluorine content of enamel layer by layer. Whether the teeth came from fluoridated or nonfluoridated cities, the concentration of fluorides was greatest on the surface, decreasing in deeper layers. The concentration of the fluorides at the surface layers appeared to continue to increase with age, even in a low fluoride community. The authors postulate that fluorides are deposited in small amounts during enamel calcification, that after calcification, but before eruption, the outer layers continue to pick up fluorides from the tissue fluids, and that after eruption the external layer may continue to get more fluoride from the oral fluids, resulting in thickening of the fluoride-saturated outer layer. The fluoride concentration in the outer layer of enamel varied from 331 ppm in incompletely formed unerupted teeth from a low fluoride area, through 1,247 ppm in the over-50-year-age group to 1,859 ppm in mottled enamel. Only the outer layers of enamel were considered to have enough fluoride to affect the carious process.

Brudevold and Steadman (p. 430) found that lead has a similar distribution in enamel to that of fluorine suggesting similar preeruptive and posteruptive mechanisms of uptake. The concentration of lead at the outer layer may be twenty times that of the whole enamel.

Clegg and Rae (p. 438) have found that the quantity of glucose added to saliva had little effect on ammonia production, 0.1 to 10 per cent glucose producing comparable results. Wide variations in urea concentration (0.1 to 5 per cent) resulted in comparable ammonia production but 10 per cent urea inhibited ammonia production. The ratio of glucose to urea appears critical in inhibiting ammonium production. The variations resulting in these in vitro studies suggest some of the complications of attempting to control ammonium production, intraorally, by adding small quantities of ammonia and urea in various agents.

Tooth germ cultivation in vitro has been the subject of investigation under a U.S.P.H.S.

grant. The latest phase of this study, reported by Lefkowitz and Swayne (p. 440), indicates that the explanted material may carry with it enough nutrient materials for about 4 days' growth (and 1 day of in vivo-equivalent-development). For development beyond this limited time it appears that the material, such as that available in embryo extract, is necessary. It is conceivable that synthetic substitutes for embryo extracts may be developed so that more precise studies of the relative roles of various inorganic and organic factors may be evaluated.

Korchin (p. 446), in the paper that won the Novice Award at the 1954 meeting, describes the effect of starch sponge in experimental animals and in human subjects. Although residual particles may remain in the bone marrow the starch sponge does not interfere with osteogenesis in the tooth socket or bone wounds. However, probably due to breakdown of the starch and secondary infection, there is acute inflammation in the dog tooth socket and delayed healing in both dogs and human subjects.

Swartz and Phillips (p. 458) point out that the residual mercury content of amalgam restorations is not critical in relationship to strength at levels below 55 per cent residuum. However, this appears to be an important point, and amalgams, or regions of amalgam restorations, containing more than this amount of residual mercury are seriously weakened. Analyses of portions of restorations showed marked variation in residual mercury content between various regions of a single restoration. Since the original mercury-alloy ratio and the condensing pressure control the residual mercury level, and thereby the strength, the operator has the problem within his control.

Kreshover and Hancock (p. 467) induced lymphatic choriomeningitis in pregnant mice and studied the effect on the fetus and on the maternal and offspring's teeth. The so-called placental barrier did not prevent infection of the fetus, especially if the virus was injected into the mother early in pregnancy. Dental defects were produced in the young of infected mothers, more frequently in those offspring that had shown virus in the blood. It is not surprising that the enamel organ defects were found in the uninfected young of infected mothers as well as in the infected young, for anything (including the mothers' metabolic state) that affects the metabolic activity of the ameloblasts may result in enamel hypoplasia.

Dienstein (p. 479), from a histologic study of the jaws of ten rats from birth to 31 days of age, made a biometric analysis of the relationship of the mandibular first molar root to the lower border of the mandible, i.e., attempted to contribute to the knowledge of tooth eruption. The apex of the tooth gradually moves away from the lower border of the mandible, and the growth of the mandible in height appears to be completed when tooth root formation terminated. Jaw growth and development relationships to tooth eruption apparently cease when root formation is complete.

Levey (p. 491) describes a specific stain for cementum. As stated, it is not histochemically specific and is empirical. However, there should be some specific relationship between the tissue and the staining agents. If cementum is chemically the same tissue as given in histological description, i.e., a modified bone formed on the tooth root, the staining effect may be the result of the great density of cementum but the stain should then react similarly on the sclerotic bone.

H. B. G. R.

Word of the death of Dr. W. J. Gies, Editor Emeritus of the JOURNAL, has just been received. All associated with dental research recognize his many contributions and mourn his passing.

EVALUATION OF LABORATORY TESTS FOR THE ESTIMATION OF CARIES ACTIVITY

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A NUMBER of laboratory procedures which utilize saliva as the test material have been advocated for the estimation of caries activity. The accuracy of any of these methods in predicting qualitative or quantitative clinical change remains unsettled. We proposed, therefore, to evaluate as many of these tests as practicable by means of frequent sampling of a group of children for the laboratory data and comparing the results with their clinical experience measured semiannually over a 5-year period. The present papers cover the classification, selection, and development of laboratory tests for study as well as comparison with each other where sufficient data warrant. Difficulties encountered in recording and assessing caries experience, especially in children with mixed dentitions, have delayed publication concerning correlation of laboratory findings with actual clinical change.

A detailed analysis of the literature did not seem necessary when digests or summaries of practically every article related to the subject have appeared in the two current surveys of dental caries literature,[1, 2] or partially reviewed by Snyder[3] in comparison with an "ideal" laboratory test for office practice. Therefore, only a listing of the type of laboratory tests is given, with supplementary information about choice of tests and other comparative studies. These may be classified as follows:

A. Bacteriologic
   1. Lactobacillus count
   2. Acid production in selective culture media
   3. Acid production in glucose or sucrose

B. Salivary Component or Property
   1. pH
   2. NH_3
   3. Ca/P
   4. Buffering capacity
   5. CO_2 capacity
   6. Amylase

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Selection of the tests employed in this longitudinal study has depended upon the status of a given test in clinical practice, the promise of a procedure, and adaptability for routine analysis of relatively large numbers of specimens.

Of the bacteriologic technics, the lactobacillus count as developed by Hadley[4] has been the most widely used and accepted by the dental profession, but maximum correlation with clinical change has seldom exceeded 90 per cent. The tomato-juice peptone agar (pH 5.0) used for this purpose can now be replaced by an essentially synthetic medium described by Rogosa, Mitchell, and Wiseman.[5] Rate of acid production in selective culture media adjusted to pH 5.0-5.4 containing appropriate indicators is closely related to numbers of lactobacilli.[6-8] Davies, Tilden, and Slack[9] used a glucose-dicalcium phosphate medium (pH 7.0) in which the dissolution of the phosphate around the colonies not only differentiated the strong from the weakly acidogenic bacteria but also allowed an estimation of numbers of these types. It is quite feasible to conduct any or all of these bacteriologic tests on 200 specimens of saliva in a working day.

The amount of acid produced in glucose or sucrose solutions in 4 hours by the action of the bacterial flora of the saliva has been measured in terms of powdered enamel dissolved,[11] pH change,[12, 13] and alkali required to neutralize it.[14] All of the authors concerned have reported good to excellent correlation with caries activity (85-91 per cent). As with the bacteriologic technics, these tests can be done together on freshly collected specimens of saliva, but the technical difficulties of the Fosdick technic[11] rule it out for large scale study.

There is little evidence that either pH, NH_3, or the Ca/P ratio is in any way correlated with caries activity. General agreement[15-18] seems to exist that saliva from the caries-resistant has a higher buffering, CO_2, and acid neutralizing capacity than saliva from caries-active subjects. These tests usually require completion within minutes or an hour. Since the time to collect and transport the specimens of saliva to the laboratory exceeded 1 hour, attempts were made to control carbonate exchange by prompt cooling in an insulated box containing dry ice, but the temperatures involved (–5° C.) markedly influenced the lactobacillus count and salivary amylase activity. Collection under oil was objectionable for plating work in bacteriology. Since it was impractical to do buffering and CO_2 capacity tests with the bacteriologic and salivary amylase tests; the former have not been included. The status of salivary amylase remains controversial.[19-22] The development of a satisfactory quantitative amylase test suitable for large numbers of specimens permitted evaluation of the role of amylase along with the bacteriologic methods.

Comparison of laboratory data with DMF or clinical experience in measured time is usually limited to a given test; comparison of laboratory tests with each other is rare. Some authors have included a second laboratory test, usually the lactobacillus count, with the one under investigation. A few have used more than 2. Thus, Dewar[23] and Kesel[24] studied lactobacillus count, calcium dissolution time, as well as pH change and titratable acidity (buffering capacity and Wach's test, respectively). Excellent correlation with caries activity was obtained with lactobacillus count, enamel dissolution time, and pH change but not titratable acidity.[14] Davies, Tilden, and Slack[9] and Rizzo

and Tilden[10] compared lactobacillus counts, Snyder test, and their special medium, which differentiated strong from weakly acidogenic bacterial, with clinical change occurring over 1 and 2 years. Again, there was a high degree of correlation with the lactobacillus count and the acidogenic types with clinical change. The Snyder test seemed to have more validity in predicting clinical change than the other two. Rickles[13] compared the pH as estimated colorimetrically, titratable acidity in sucrose solutions, and lactobacillus counts for the prediction of new surfaces occurring 1 year later. He did not compare these procedures with each other, but there was a definite correlation between all 3 methods and the amount of new caries experience. In contrast, Boyd, Cheyne, and Wessels[25] and Glass[26] could find no relationship between lactobacillus count and caries experience. Rae and Clegg[27] tested, but could find no relationship between buffering capacity, viscosity, and lactobacillus count of saliva. Sullivan and Storvick[28, 29] analyzed and correlated each of 6 laboratory tests (pH, NH_3 buffering capacity, amylase, lactobacillus count, and Snyder test) with each other and the DMF records of a group of college freshman and another of teen-age children. The most significant correlations were obtained for buffering capacity, amylase, lactobacillus count, and Snyder test. In our opinion the comparison of laboratory data which represent present findings with DMF or past change is invalid because the "F" portion of the index frequently includes events of many years' standing. On these grounds much of the early work on amylase, the results reported by Marshall-Day, Shourie, Hein, Leung, and Simmons,[30] Sullivan and Storvick[28, 29] and others can be eliminated from serious consideration.

As stated previously, the primary objective was to evaluate tests in parallel with caries activity measured over what we considered a minimal period (5 years). The finding of a satisfactory "captive" population without an abnormal rate of loss was a problem, but nearby Schools for the Deaf offered reasonably stable groups because of the time needed to master communication. In addition, a local Indian School had a group of Navajos of high school age sent each year for 5 years of specialized training. We initiated studies at the State Schools for the Deaf in Oregon and Washington as well as Chemawa Indian School, but circumstances somewhat beyond our control necessitated a choice between annual clinical examination of larger numbers of children without supporting radiographic records or smaller numbers examined clinically every 6 months with radiologic studies included. The second alternative was chosen: The children from one school (Washington State School for the Deaf) were selected for the clinical study. Although the fourth clinical examination covering 2 full years was recently finished, the tabulation of the clinical findings for the highly desirable correlation with laboratory data has not been completed. Meanwhile it seemed of interest to compare the results obtained with the different laboratory tests employed: lactobacillus count, acid production in selective media, and salivary amylase.

EVALUATION AND COMPARISON OF LABORATORY TESTS

A. *The Lactobacillus Count.*—As it was originally described,[4] the technic of the lactobacillus count consisted of chewing on a piece of paraffin to collect

2 to 8 ml. of saliva, which was then diluted to 10.0 ml. with sterile physiologic saline; the specimen was shaken by hand for 3 minutes, and 1-5 and 1-20 dilutions made to allow for low and high counts. Of these dilutions, 0.1 ml. samples were spread over the surfaces of tomato-juice peptone agar plates (pH 5.0). Counts made after 3 to 4 days' incubation represented the number of lactobacilli per milliliter in the constant volume of the first dilution. This was modified[31] so that 1.0 ml. of saliva was added to 4.0 ml. of acid glucose infusion broth (pH 5.0) to give a 1:5 dilution of the original specimen and 0.1 of this dilution spread over the medium. The purpose of the acid glucose broth was to furnish a qualitative answer for the presence or absence of gram-positive bacilli, assumed to be lactobacilli, when the corresponding plate cultures were negative (less than 50 lactobacilli per milliliter of saliva). When negative or low counts were anticipated, 0.1 to 0.4 ml. of saliva could be used. The procedure has remained essentially unaltered. Criticisms of the technic have centered about the crudity of the medium, failure to indicate physiologic behavior of the organisms cultured, overgrowth of plates by yeast forms and mold contaminants, length of incubation, and statistical inaccuracies.

The tomato-juice peptone agar, hereafter referred to as TJA, is undoubtedly crude compared with the nearly synthetic medium (SLA).[5] The filtrate of canned tomatoes with added peptone and peptonized milk is a nutritional unknown; it is translucent and without a selective inhibitor mold contaminants or yeast forms of the saliva readily overgrow the plates. It is also preferable to make TJA in the laboratory. In contrast, the SLA formula[8] represents the result of a careful study of the nutritional requirements of the lactobocilli; it is clear and so highly selective that it need not be autoclaved for dispensing or even storage. Neither medium provides any information about the physiologic behavior of the organisms except their ability to grow at pH 5.0-5.4 and both require 3 to 5 days' incubation for counting purposes.

The statistical inaccuracies of the lactobacillus count are derived from several sources: distribution of the organisms in the saliva, growth under conditions imposed, and the actual count. Under the heading of distribution in the saliva come the variables of hour and time required for collection and dispersion in a medium of fluctuating viscosity. There seems to be little question that the highest numbers of lactobacilli occur in specimens of saliva collected before breakfast, but Sandy[32] found no significant differences in the variations of counts obtained from a group of specimens collected at 10:00 A.M. over those collected at 7:00 A.M. This would indicate that collection should be made as far as practicable at a uniform time. Collection also requires the co-operation of the subject to chew the paraffin so as to cover all the exposed surfaces and collect all the saliva obtained in a 3- or 5-minute period. This means there is an age period (6 years) below which reliable or representative samples are difficult to get. The organisms may be unequally dispersed in a medium varying widely in respect to food debris, viscosity, and volume.[33] Although the lactobacilli have no cytochromes and thereby are similar to the anaerobes,[34] most oral strains grow microaerophilically or quite well under atmospheric conditions normally prevailing for lactobacillus counts. Ericsson and Hagglund[35] called attention to the increased counts they were able to

obtain anaerobically compared with aerobic counts for the same specimens of saliva, yet their conclusions were based on a very small number of samples. It is, nevertheless, possible that poured plates would provide better oxygen tensions for growth of the oral lactobacilli but unless one assumes all colonies to be lactobacilli, the technical aspect of microscopic differentiation of colonies even under low magnification (7×) is a serious objection in routine practice. Finally, there is the statistical accuracy of lactobacillus counts. Unlike milk or water bacteriologic counts where a series of dilutions are made in duplicate or even triplicate for statistical desirability, a lactobacillus count is the growth of organisms from a single dilution of saliva on one plate of highly selective medium. The number of colonies may range from 0 to 1,000,000 or more. Lewis and Hartman[36] found by carefully standardizing their technic an excellent consistency was attained not only by one worker counting results from different specimens of saliva but also from the same specimens counted by several individuals. They felt that with an initial 1-10 dilution of the saliva in saline and 0.1 ml. of that dilution cultured, the most accurate estimates were between 20,000 and 90,000 lactobacilli per milliliter of saliva (200 to 900 colonies per plate). On the other hand, as Sandy[32] and others have maintained, there is little reliability in a single count for clinical interpretation.

B. *Acid Production in Selective Culture Media.*—A great shortcoming of the lactobacillus count is that 3 to 5 days must elapse before an answer can be given. Broth or fluid media give obvious and prompt answers about acid production but have the disadvantage of usually not allowing estimates of numbers of specific bacteria introduced. To meet this objection Snyder[3] suggested the addition of agar to the acid glucose broth (pH 5.0) to provide dispersion of colonies as in a "shake" tube and the indicator, bromcresol green, to reflect acid production. This medium proved very satisfactory since the rate of acid production could be daily observed. Acid formation was caused primarily by growth of the lactobacilli and the rate was generally proportional to their numbers. On the basis of positive color changes in 24 and 48 hours as indicative of marked to moderate caries activity and little or no change in 48 to 72 hours as evidence of slight or negative caries activity, a high degree of correlation was found in a group of 66 children studied over a 2-year period.[7] Although the relation of color change in the medium to the oral lactobacilli has been supported,[37, 38] sufficient confirmation of its capacity as an index of caries activity has not been made. Nevertheless, the Snyder test, as it is now called, has been widely employed for a susceptibility test in state health laboratories, dental clinics, and office practice. Rogosa and Wiseman[8] recently proposed for test tube purposes a modification of the SLA which included alizarin red S as the indicator; growth and subsequent acid production with color alteration occurred rapidly from small inocula of lactobacilli. They also claimed the color ranges could be interpreted directly in terms of numbers of lactobacilli originally introduced.

MATERIALS AND METHODS

A. *Subjects.*—The children under investigation resided during the school year in the State Schools for the Deaf of Oregon and Washington and the

Chemawa Indian School in Salem, Oregon. The age of the group study ranged from 6 to 16 years. The number of subjects was 1951-1952: 190; 1952-1953: 550; 1953-1954: 150.

B. *Collection Schedule.*—It was impracticable to obtain prebreakfast specimens in any of these schools or during the morning for the Navajo group at Chemawa. However, the collections were always made at the same time for each school or group:

Oregon School for the Deaf: 9:30-11:30 A.M.; Washington School for the Deaf: 9:00-11:00 A.M.; Chemawa Indian School, Elementary: 9:30-11:30 A.M.; Secondary (Navajo): 1:30-3:30 P.M.

C. *Time for Collection of Specimen.*—Three to five minutes of chewing paraffin tablet according to directions given. This remained a weak spot since it was difficult to control when collecting in groups of any size and also for the youngest children of a handicapped group where communication was basically one of demonstration. It was the hope, however, that multiple samples from each child would offset much of the error incurred.

D. *Preservation of the Specimens.*—At first, no attempt was made to keep the specimens of saliva other than at environmental temperatures. Because of refrigeration requirements for other tests, an insulated box was used during the 1953-1954 school year. When dry ice slabs were used in this box, the specimens of saliva froze and this effect was deleterious to both lactobacillus counts and salivary amylase tests. Resort was made to slabs of frozen plastic gels (Sno-Gel) or several units of Kand Ice placed in the box before departure. Specimens of saliva were placed in the cooled box as soon as practicable. The temperature of the box was 8° to 10° C. on return. The specimens were then stored at 5° C. in the refrigerator until cultured or analyzed. Experiments showed that the lactobacillus counts were stable at room temperature for 4 days and in the icebox for 3 days. Since all our specimens were cultured within 24 hours of collection, it was concluded that storage at refrigerator or room temperature had no effect on these lactobacillus counts.

E. *Culture Media.*—1. Tomato-juice peptone agar (pH 5.0) was prepared by the formula given by Jay.[31] Adjustment of 10 liter volume lots to pH 4.8-5.0 varied from the simple addition of lactic acid (1.0 ml.) to neutralization with 40 per cent NaOH and acidification to pH 5.0 with undiluted lactic acid. In either case, the purpose seemed to be to provide lactate, the effective range of which Dewar[39] found to be between a 1:175 and 1:300 concentration (5.6-3.3 grams per liter). In our experience the amount of lactate was not critical as long as some was present and the pH 4.8-5.0. Additional selectivity in respect to molds and yeasts was made possible by the addition of sodium azide (1 per cent) as suggested by Diamond[40] to give a final concentration of 1:10,000. Dewar[39] objected to the use of the azide on the grounds of inhibition of certain types of lactobacilli, while Rogosa and Mitchell[41] showed the azide bound an essential nutrient, magnesium, for the lactobacilli. On the other hand, Eriesson and Hagglund[35] found no differences in lactobacillus counts on TJA

with and without azide, while Snyder and Slawson[42] in studying the effects of azide and pH in a series of 600 specimens of saliva plated on TJA with azide (1:10,000) and SLA found the following distribution: similar lactobacillus counts, 43 per cent; higher on TJA with azide, 12 per cent; higher on SLA, 45 per cent. However, since all specimens could be cultured and counted on the TJA with azide, it was concluded that this gain more than offset the loss resulting from overgrowths on TJA without azide by mold (air contaminants) or the yeastlike forms of the oral flora.

2. SL agar was prepared by Rogosa's formula. Our experience showed the medium needed to be made more selective. This was done by dropping the pH from 5.4 to 5.2 with additional glacial acetic acid. However, the dehydrated product (Difco) was satisfactory without further alteration.

3. Snyder Test Agar was prepared from the dehydrated medium (Difco). Some lots were too acid and required adjustment to pH 5.0 with N/1 NaOH. It was also found desirable to add another 10 mg. of the indicator (bromcresol green) per liter for optimal reading of color change.

4. SL agar (modified). Rogosa and Wiseman[8] modified SL agar by adding of 0.05 per cent arabinose and 0.004 per cent alizarin red S, and reducing the agar content to 0.2 per cent. We prepared this medium accordingly.

E. *Procedure.*—The specimens of saliva were held in the refrigerator until used. They were shaken in lots of 9-10 at a time for 5 minutes into a Kahn shaking machine after which 0.2 ml. of each specimen was pipetted in melted and cooled (50° C.) Snyder test agar and 0.5 to 4.5 ml. diluent (phenol red glucose broth or water). Distribution from the latter was made as follows: 0.1 ml. to TJA plate, 0.1 ml. to SLA plate, 0.5 ml. to SLA (mod.) tube. The TJA and SLA plates were incubated 4-5 days at 37° C. Counts were made for the most part by one of us (M. K. G.) using a binocular stereoscopic microscope (7×) and a Frost counting card (No. 1099). Enough checks were made to assure an accuracy similar to that described by Lewis and Hartman.[36] The tubes of media reflecting acid production were also incubated at 37° C. but were read daily by one of us (M. L. S.)

RESULTS

I. *Lactobacillus Counts on TJA and SLA.*—Lactobacillus counts were made from the beginning of the study on TJA containing sodium azide in a 1:10,000 concentration and somewhat later on SLA. The counts for specimens of saliva cultured on the respective media were recorded as 3 place logarithms on IBM punch cards for comparative purposes. Thus, the figures obtained represent the total lactobacillus counts and do not take into account the problem of colony type or the significance, if any, of any one type. For that matter, colony variation was much easier ascertained on TJA than SLA. Summation of the results was in terms of the characteristics of the logarithmic values; these are listed in Table I.

TABLE I
COMPARISON OF LACTOBACILLUS COUNTS OBTAINED FOR SPECIMENS OF SALIVA CULTURED, RESPECTIVELY, ON TJA CONTAINING 1:10,000 SODIUM AZIDE AND SLA

LOG LBC SLA	NO. SPEC.	LOGARITHMIC VALUES OF LACTOBACILLUS COUNTS ON TJA WITH 1:10,000 AZIDE									
		0-2		2-3		3-4		4-5		5-6	
		NO.	PER CENT	NO.	PER CENT	NO.	PER CENT	NO.	PER CENT	NO.	PER CENT
0-2	1,435	1,234	86*	127	9	57	4	14	1		
2-3	463	140	29	153	43	60	16	9	3		
3-4	564	68	12	104	18	324	57	65	12		
4-5	981	15	2	25	4	165	17	663	71	68	7
5-6	323	3	1	1		9	3	140	35	196	61
Total	3,666										

*Calculated to nearest per cent.

Table I shows the differences between lactobacillus counts obtained on TJA with azide (1:10,000) and SLA of our preparation. Over the entire range counts were lower in 2 to 37 per cent and higher in 9 to 20 per cent of specimens of saliva cultured on the SLA compared with TJA; the best matching values occurred at the zero and highest counts. That this discrepancy may be attributed in part at least to the azide rather than the superiority of the SLA is shown in Table II where the azide concentration was lowered to 1:20,000.

TABLE II
COMPARISON OF LACTOBACILLUS COUNTS OF SPECIMEN OF SALIVA CULTURED ON SLA AND TJA WITH SODIUM AZIDE IN 1:20,000 CONCENTRATION

LOG LBC ROGOSA	SPECIMENS		LOGARITHMIC VALUES OF LACTOBACILLUS COUNTS ON TJA WITH SODIUM AZIDE (1:20,000)									
			0-2		2-3		3-4		4-5		5-6	
	NO.	PER CENT	NO.	PER CENT	NO.	PER CENT	NO.	PER CENT	NO.	PER CENT	NO.	PER CENT
0-2	121	30.3	105	87	9		5	4	2	2		
2-3	41	10.0	11	27	16	39.0	8	20	6	15		
3-4	74	18.4	3	4	11	5.0	40	54	19	24	1	
4-5	122	30.3	2	2	0		13	11	80	66	27	22
5-6	45	11.1	0		0		0		4	9	41	91
Total	403											

In Table II the deviations below and above the duplicating values for lactobacillus counts on TJA containing 1:20,000 sodium azide compared with those on SLA were 9-27 per cent and 14-35 per cent, respectively. That is, there were roughly as many lower as higher, which would bring the results within variations expected for these plating procedures.

Since the number of specimens of saliva cultured on TJA containing 1:20,000 sodium azide was small compared to those on SLA and TJA with 1:10,000 sodium azide, it seemed of interest to examine the distribution of lactobacillus counts on these 3 media. Because of problems peculiar to the Chemawa Indian School, the figures presented in Table III were derived only from the 2 Schools for the Deaf.

The impressive feature of Table III is the surprising constancy in the distribution of lactobacillus counts obtained over a 2-year period from these

TABLE III
DISTRIBUTION OF LACTOBACILLUS COUNTS FOR SPECIMENS OF SALIVA CULTURED ON SLA AND TJA WITH 1:10,000 AND 1:20,000 CONCENTRATIONS OF SODIUM AZIDE

	SLA		TJA			
			1:10,000 NaN_3		1:20,000 NaN_3	
LOG LBC	NO. SPEC.	PER CENT TOTAL	NO. SPEC.	PER CENT TOTAL	NO. SPEC.	PER CENT TOTAL
0-2	656	31.1	1,442	32.2	121	30.3
2-3	213	10.1	460	10.4	41	10.0
3-4	335	15.9	700	15.9	74	18.4
4-5	636	30.2	1,198	27.1	122	30.3
5-6	267	12.8	542	12.3	45	11.0
Total	2,108	100.1	4,415	100.1	403	100.0

children. The maximum difference between lactobacillus counts made with these 3 media for any logarithmic values listed is 3.2 per cent. It would appear, therefore, that the conclusions reached with the sample employed (403 specimens) were valid.

II. *Acid Production in Selective Culture Media Compared With Lactobacillus Counts.*—Snyder test agar was used throughout the investigation with a uniform inoculum of 0.2 ml. saliva. The SLA (mod.) was introduced later and, after the usual preliminary study of a new medium, the inoculum was standardized at 0.05 ml. of the saliva (0.5 ml. of the 1-10 dilution). In Snyder test agar the indicator, bromcresol green, is blue-green at pH 5.0, green at pH 4.5-4.7, and yellow at pH 3.8-4.0. On a 4 plus scale, these represent 0, +, and ++++ changes, respectively. There is no difficulty about the extremes, but an opportunity exists for variation as the yellow becomes increasingly dominant (pH 4.4-3.8). This is particularly true when large numbers of specimens are examined in a test tube rack; for example, in 24 hours the blue-green range is dominant and one readily notes a change toward the yellow which may be recorded as ++, but the same tube may not change any more. Whereas in 48 hours, when the majority of tubes has changed completely, this tube will stand out as sufficiently green to cause some puzzlement about its position. The indicator in the SLA (mod.) is alizarin red S which is red purple at pH 5.6 and yellow at pH 4.2 with gradations going through the purple and orange with a rather sharp change at pH 4.7. Although a purple color is visually preferable to the green, the changes still occur in gradations which are difficult to decide and there is frequent reversion upon standing. Again, recordings were made on a 4 plus scale. With both media any significant changes were recorded as ++, and for summary purposes included as positive findings (++, +++, ++++). The comparison of these color changes in the 2 media in respect to time with the lactobacillus counts of respective specimens of saliva cultured on TJA and SLA is made in Tables IV and V.

Analysis of these tables shows the clear correlation between rate of color change in these media and increasing numbers of lactobacilli. It should be pointed out that the percentage of positive reactions in 24 hours is considerably higher with lactobacillus counts in the log 2-4 categories in this study than in

previous reports.[7, 43] Under the conditions of this investigation, however, the results indicate there is little to choose between the 2 media in terms of rate of color change obtained with the inocula employed and the respective lactobacilli counts. The maximum difference between the 2 tests for any of the listed logarithmic values of lactobacillus counts on either SLA or TJA was 10 per cent and in two-thirds of the daily readings the difference was 3 per cent or less. In our opinion, this would leave the investigator free to choose whichever of these media would appear suitable for the problem at hand.

TABLE IV

COMPARISON OF PERCENTAGES OF POSITIVE COLOR REACTIONS IN 24, 48, AND 72 HOURS IN SNYDER AND ROGOSA INDICATOR MEDIA WITH LACTOBACILLUS COUNTS OBTAINED ON TJA WITH 1:10,000 SODIUM AZIDE FROM RESPECTIVE SPECIMENS OF SALIVA

LOG LBC T. J. A.	NO. SPEC.	COLOR TEST	REACTIONS					
			24 HOUR		48 HOUR		72 HOUR	
			PER CENT POS.	DIFF. PER CENT	PER CENT POS.	DIFF. PER CENT	PER CENT POS.	DIFF. PER CENT
0-2	2,356	Snyder	5.0	2	16.0	3	31	3
	1,470	Rogosa	3.0		19.0		34	
2-3	700	Snyder	13.0	10	59.0	3	81	8
	379	Rogosa	23.0		56.0		73	
3-4	964	Snyder	25.0	5	82.0	1	91	4
	582	Rogosa	20.0		83.0		87	
4-5	1,405	Snyder	52.0	3	94.0	2	97	3
	931	Rogosa	49.0		92.0		94	
5-6	1,030	Snyder	74.0	10	99.0	3	99	3
	323	Rogosa	64.0		96.0		96	

TABLE V

COMPARISON OF PERCENTAGES OF POSITIVE COLOR REACTIONS IN 24, 48, AND 72 HOURS IN SNYDER AND ROGOSA COLOR MEDIUM WITH LACTOBACILLUS COUNTS OBTAINED ON ROGOSA'S SL MEDIUM FROM RESPECTIVE SPECIMENS OF SALIVA

LOG LBC ROGOSA SL AGAR	NO. SPEC. SALIVA	COLOR TEST	REACTIONS					
			24 HOUR		48 HOUR		72 HOUR	
			PER CENT POS.	DIFF. PER CENT	PER CENT POS.	DIFF. PER CENT	POS. PER CENT	DIFF. PER CENT
0-2	1,470	Snyder	7	2	16	2	24	10
	1,470	Rogosa	5		18		34	
2-3	379	Snyder	11	4	49	5	67	7
	379	Rogosa	7		54		74	
3-4	582	Snyder	19	0	76	3	89	2
	582	Rogosa	19		79		87	
4-5	931	Snyder	47	9	91	0	95	2
	931	Rogosa	38		91		93	
5-6	323	Snyder	75	5	98	3	99	2
	323	Rogosa	70		95		97	

DISCUSSION

Although it is recognized that the real core of this project is correlation of the results of the various laboratory tests with actual clinical change, it seemed useful to ascertain variations or preference of culture media proposed

for bacteriologic tests. The results obtained for lactobacillus counts indicated SLA was somewhat superior to TJA if sodium azide was used in a 1:10,000 concentration in the latter, whereas with acid production in selective culture media the percentage of positive reaction in Snyder test agar and SLA (mod.) were essentially the same for listed lactobacillus count ranges and day of observation. On the other hand, when the azide was decreased to a 1:20,000 concentration, the lactobacillus counts were frequently higher on TJA than SLA. This would refute to some extent the objections raised[39, 41] to the incorporation of sodium azide in TJA used for lactobacillus counts. Certainly, the use of that agent in this concentration makes the TJA as selective a culture medium for lactobacillus counts as the more truly synthetic SLA and with the advantage of selecting colonial variants. The investigator now has a choice of selective culture media, each one of which will give results not significantly different from the other in respect to lactobacillus counts or acid production.

SUMMARY

Lactobacillus counts made on tomato-juice peptone agar (pH 5.0) containing sodium azide as a selective inhibitor were comparable with respective counts on Rogosa's SL agar if the azide was present in a 1:20,000 concentration. No essential difference was observed between the rate of color or acid formation in either Snyder test agar or Rogosa's SLA (mod.) medium compared with the lactobacillus counts of respective specimens of saliva.

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THE INCIDENCE OF DENTAL CARIES IN ADOLESCENTS WITH DIFFERENT OCCLUSION

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SEVERAL items were reported which were examined for their influence upon the incidence of dental decay. Surprisingly, no reliable data seem to exist correlating arrangement and occlusion of the teeth with the incidence of dental caries. It is generally accepted that persons with maloccluding dentitions are more prone to decay than those with a perfect occlusion. This unsupported belief is one of the main indications of orthodontic treatment from a medical point of view. However, no data were found in the literature accessible to me, including the most recent textbooks and reviews, on the relative incidence of caries in the different types of malocclusion.

An attempt was made to fill this gap in our knowledge by examining representative samples of adolescents of both sexes with regard to the incidence of decay, in connection with normalcy of occlusion and/or type of malocclusion. Results of these studies are reported briefly.

MATERIAL AND METHODS

The total grammar school population (all over 14 years old) was examined once in 5 Hungarian cities (Debrecen, Sátoraljaújhely, Gyöngyös, Eger, and Szekszárd) during the years 1951 to 1954. Data of the 16- to 18-year-olds were selected for this study.

Pupils were examined in the classrooms, independently, by two examiners. One dictated the caries records (teeth present, fillings, crowns, decay needing treatment or extraction) to a clerical assistant; the other examiner classified pupils with regard to normalcy of occlusion or presence and type of malocclusion, adhering to a simple diagnostic scheme. Classification of the pupils into any of the occlusion types is self-explanatory, generally. Minor divergencies from what is regarded as perfect (ideal) occlusion were still regarded as normal, if no functional or cosmetic disturbance needing orthodontic treatment was seen. Class I, Class II, Divisions 1 and 2, and Class III malocclusion (including subdivisions) were classified according to Angle. In addition, two separate groups were formed: one group of persons displaying excessive overbite due to retruded upper incisors with intermaxillary molar and canine relationships as in Class I (covered bite); the other of persons with mutilated dentitions in which either several teeth were lost and occlusion of the remaining ones could not be judged reliably or in which after the loss even of a single tooth (mostly of at least two teeth) shifting of the remaining ones occurred, causing a disturbance of the pre-existing pit-fissure occlusion pattern.

Caries data were evaluated on the DMF tooth basis using the symbol CER[1] (initials of Latin words instead of English ones: Caries for decayed, Extractio for missing, and Restauratio for filled teeth, including crown work of all types).

The cities of Debrecen, Sátoraljaújhely, Gyöngyös, and Eger are supplied with domestic waters containing low fluoride levels, whereas in the domestic water at Szekszárd the fluoride level amounts to approximately 0.75 ppm. For this reason, data of the pupils attending

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the grammar schools at Szekszárd are separated from the others. The caries protective action of the domestic water of Szekszárd has been shown by comparing the caries data of pupils born and continuously resident with all others and with caries data of the grammar school population of two low fluoride cities (Adler and Gergely[2]). Since, in the present paper, our aim is to show eventual differences in the incidence of decay depending upon normalcy of occlusion or type of malocclusion, no distinction is made between pupils born and continuously resident and others in any of the cities. The grammar school population of Debrecen, Sátoraljaújhely, and Gyöngyös was examined and the combined results of these 3 low fluoride cities were subjected to statistical analysis. Having found typical differences, as a control measure examinations were carried out at Eger (a low fluoride city where artificial fluoridation of the domestic water is planned) in the early spring of 1954. The data of this examination series are handled separately, although they could be combined with those of the other 3 low fluoride communities.

RESULTS

The combined data for both sexes are shown in Table I for Debrecen, Gyöngyös, and Sátoraljaújhely, for Eger, and for Szekszárd. The number of subjects is shown, the range of the CER (DMF) values, the mean CER count with its standard error, and the standard deviation.

While in most types of malocclusion the mean of the CER index is higher than in normal occlusion, in all 3 groups the CER index of persons with covered bite, Class I is markedly lower. A similar arrangement of the incisors is typical for most cases of Class II, Division 2 malocclusion. Although in the combined data of the 3 cities this type of malocclusion is associated with a slightly higher CER index than the normal occlusion group, at Eger as well as at Szekszárd a lower index was observed in this type of malocclusion than in the normal occlusion group. The incidence of caries is, nevertheless, in all instances greater in the Class II, Division 2 malocclusion type than in the covered bite, Class I group. These findings deserve special attention because they demonstrate certain types of malocclusion with incidence of less (or at least not more) decay than in normal occlusion.

The highest index values were observed quite uniformly in the "mutilated dentitions" group. While in all other types of occlusions the CER values were distributed over a broad range starting with 0, in this group, by definition, the starting point was CER 1.

Although at the age of 16 to 18 years there is a well-marked difference in the incidence of caries between boys and girls, in Table I data for both sexes are combined, in order to increase the number of the subjects in the single subgroups. However, differences of similar range are obvious from Table II where data for boys and girls were computed as the mean of the CER indexes, in the combined 3 cities group and at Eger.

Statistical significance tests were made first of all in the combined male and female population of the 3 cities group. A modification of the chi square test, as described by Cramér, was used upon the suggestion of B. Gyires of the Institute for Applied Mathematics of the Hungarian Academy of Sciences, Division Debrecen. This test permits the comparison of 2 groups with irregular

TABLE I

THE INCIDENCE OF DECAY IN ADOLESCENTS WITH DIFFERENT OCCLUSION

OCCLUSION	DEBRECEN, GYÖNGYÖS, AND SÁTORALJAÚJHELY				EGER				SZEKSZÁRD			
		CER (DMF) TEETH				CER (DMF) TEETH				CER (DMF) TEETH		
	NO.	RANGE	M ± ε	S.D.	NO.	RANGE	M ± ε	S.D.	NO.	RANGE	M ± ε	S.D.
Normal	1,657	0-21	3.60 ± 0.075	3.06	490	0-18	3.48 ± 0.17	3.74	298	0-21	2.39 ± 0.17	2.96
Cl. I., Angle	581	0-17	4.40 ± 0.16	3.37	203	0-22	4.90 ± 0.29	4.17	79	0-20	3.48 ± 0.42	3.70
Cl. II., Div. 1 Angle	185	0-15	4.95 ± 0.29	4.38	84	0-18	4.42 ± 0.45	4.10	17	0- 9	2.94 ± 0.60	2.48
Cl. II., Div. 2 Angle	251	0-15	4.12 ± 0.23	3.60	66	0-13	3.27 ± 0.39	3.14	53	0- 9	1.87 ± 0.33	2.40
Covered bite, Cl. I., Angle	114	0- 9	2.36 ± 0.21	2.26	30	0-11	3.13 ± 0.53	2.92	51	0- 7	1.31 ± 0.25	1.80
Mutilated dentitions	478	1-22	7.38 ± 0.17	3.71	126	1-19	7.81 ± 0.33	3.72	42	1-16	6.79 ± 0.54	3.49
Cl. III., Angle	52	0-17	5.00 ± 0.63	4.06	12	0- 9	4.42 ± 0.75	2.59	7	0- 6	3.14 ± 0.85	2.24
Total	3,318	0-22	4.38 ± 0.064	3.67	1,011	0-22	4.54 ± 0.11	3.36	547	0-21	2.76 ± 0.14	3.23

M = mean, ε = standard error of the mean, S.D. = standard deviation.

distribution of the single values; it takes into consideration the number of subjects and is free from the necessity of assuming one of the observed distributions as the "expected" one. In comparing the normal occlusion group with any of malocclusion groups, we had no difficulty in assuming the normal occlusion group's distribution as the expected values, but there was no reliable criterion upon which to chose either of the two malocclusion types compared as displaying the expected distribution. For this reason, the use of this modification of the chi square test is advantageous. The working formula is:

$$\chi^2 = m\,n \sum_i \frac{1}{\mu_i + \eta_i} \left(\frac{\mu_i}{m} - \frac{\eta_i}{n} \right)^2$$

Results of the statistical significance tests are summarized in Table III. The differences of the distributions are in most instances statistically significant. Since groups are identical in every respect except the type of occlusion, we are justified in assuming that the different pattern of occlusion is the causative factor of the differences in the incidence of dental caries.

TABLE II

CER (DMF)-INDEXES OF MALES AND FEMALES (16 TO 18 YEARS OLD) WITH DIFFERENT OCCLUSIONS

OCCLUSION	THREE CITIES GROUP		EGER	
	MALES	FEMALES	MALES	FEMALES
Normal	3.3	4.0	3.3	4.3
Class I, Angle	4.2	4.7	5.0	4.8
Class II, Div. 1, Angle	4.3	5.8	3.1	5.3
Class II, Div. 2, Angle	4.1	4.2	3.6	3.0
Covered bite, Class I, Angle	2.2	2.6	3.2	3.0
Mutilated dentitions	6.7	7.7	7.5	8.0
Class III, Angle	5.0	5.0	4.9	3.8

Similar computations were made in the school populations of Eger and Szekszárd, but in most instances no significance was attributable to the differences of the distributions. This is due, however, to the small numbers of the subjects in the different occlusion groups in these cities in comparison to the combined 3 cities group.

Our observations do not offer any direct clue to the causes of the different incidence of dental decay in the different types of malocclusion. One circumstance seems, however, worthy of mention. "Covered bite" and certain forms of Class II, Division 2 malocclusion are hereditary anomalies. From our observations it seems that dentitions with retruded upper incisors are less prone to decay than those with other forms of occlusion, especially other forms of malocclusion, at least in the age group examined. Whether or not this partial resistance to decay is due to an hereditary factor or to the peculiar functioning of the masticatory system cannot be stated reliably on the basis of our present data. However, an acquired retrusion of the upper incisors is often encountered in the mutilated dentitions group, due to distal shifting of lower premolars and anteriors after the early loss of the lower first molars, and the consecutive movement of the upper incisors. The retruded position of the upper incisors determines the functional pattern in this type of occlusion; and, in all proba-

bility, whether it prevails on an hereditary or an acquired basis. In those groups with mutilated dentitions, the incidence of decay is greater than in any other, in spite of the frequency of retruded upper incisors. Thus, we are inclined to believe that the resistance to decay in the "covered bite" (and partly in the Class II, Division 2) malocclusion types is due to hereditary factors.

Comparing the combined malocclusion group with normal occlusion, the belief that malocclusion is conducive to caries is substantiated. However, it has been shown, that this generalization is not of specific validity.

The highest index values were observed in the mutilated dentions group. This group belongs to the malocclusion groups and accounts for approximately 20 per cent of all malocclueding dentitions. This fact is indicative of the important role of dental caries, not as a consequence but rather as the causative factor in certain types of malocclusion. This is also obvious from the findings of Plater.[5]

TABLE III

STATISTICAL SIGNIFICANCE OF THE DIFFERENCES BETWEEN GROUPS OF ADOLESCENTS WITH DIFFERENT OCCLUSION, TABLE I, COMBINED DATA OF DEBRECEN, GYÖNGYÖS, AND SÁTORALJAÚHELY

GROUPS COMPARED		n	χ^2	P
Normal occlusion with:				
	Class I, Angle	13	40.253	0.1
	Cl. II, Div. 1, Angle	13	38.977	0.1
	Cl. II, Div. 2, Angle	13	30.136	0.1-1.0
	Covered bite, Angle, Cl. I	7	17.976	0.1-1.0
	Mutilated dentitions	12	419.500	0.1
	Class III, Angle	9	10.668	30.0 *non*
Class I, Angle with:				
	Cl. II, Div. 1, Angle	13	9.495	70-80 *non*
	Cl. II, Div. 2, Angle	13	13.866	30-50 *non*
	Covered bite, Angle, Cl. I	7	41.210	0.1
	Mutilated dentitions	12	187.500	0.1
	Class III, Angle	9	3.41	90-95 *non*
Cl. II, Div. 1, Angle with:				
	Cl. II, Div. 2, Angle	13	24.327	2-5
	Covered bite, Angle, Cl. I	7	46.397	0.1
	Mutilated dentitions	12	84.636	0.1
	Class III, Angle	9	2.8312	95-96 *non*
Cl. II, Div. 2, Angle with:				
	Covered bite, Angle, Cl. I	7	33.392	0.1
	Mutilated dentitions	12	174.2	0.1
	Class III, Angle	9	7.733	50-70 *non*
Covered bite, Angle Cl. I. with:				
	Mutilated dentitions	6	239.27	0.1
	Class III, Angle	7	18.838	0.1-1.0
Mutilated dentitions with:				
	Class III, Angle	8	51.23	0.1

n = Degree of freedom; X^2 as computed according to the formula given in the text: P = probability on a percentage basis of how far the difference in the distribution between two groups is due to chance alone. In medical statistics, P values up to 5.0 per cent are considered as being statistically significant. When two values are shown in the column of P, the real value is between the two noticed ones. Nonsignificant differences are marked by *non*.

SUMMARY

The incidence of dental caries was examined in adolescents 16 to 18 years of age in relationship to normalcy of occlusion or type of malocclusion. Groups of individuals with malocluding dentitions display higher CER (DMF) indexes generally than those with normal occlusion. "Covered bite," with normal intermaxillary molar and canine relationships, is associated with less decay than normal occlusion. This is true to some degree for Class II, Division 2 malocclusion also, at least in so far as in this type of malocclusion decay is not heavier than in the normal occlusion group. This finding points to the role of hereditary factors in caries susceptibility. Dental decay is an important causative factor of malocclusion, approximately 20 per cent of malocclusions being apparently due to the early loss of (carious) permanent teeth.

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A SYSTEM OF CLASSIFICATION AND SCORING FOR PREVALENCE SURVEYS OF PERIODONTAL DISEASE

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THE "paucity of reliable epidemiological data"[1] concerning periodontal disease has been discussed by several commentators.[2, 3] Although field surveys of gingivitis prevalence have been carried out using the P. M. A. index of Massler and Schour,[4-10] studies dealing with more advanced stages of tissue destruction have generally been based upon collections of case histories, with all of the drawbacks inherent in such data,[11] and have ordinarily been limited to estimates of the qualitative presence or absence of disease without regard to its severity,[12] or of quantitative bone loss as determined by radiographs alone, without reference to clinical appearance.[13, 14] A committee of the University of Michigan Periodontal Workshop stated that "the lack of valid indexes for determining the prevalence and epidemiological characteristics of periodontal diseases has hindered seriously the development of more effective preventive and treatment procedures for these diseases."[1] The Council on Dental Health of the American Dental Association commented that, "The prevalence of the disease has not been measured, largely because of inadequate measuring devices."[15]

This report describes a method for scoring the effects of periodontal disease which permits quantitative comparisons between or within human populations, and which may be applied swiftly and with a minimum of equipment in the field.

METHOD AND CRITERIA

In general, the problems inherent in the formulation of an epidemiologic measure for periodontal disease parallel those which were encountered and solved in the development of an epidemiology of dental caries. In both areas the raw data employed do not represent an objective measurement (such as estimation of the pH of a solution) but rather a series of relatively subjective clinical diagnoses. Two principal difficulties arise: (a) there are wide variations in the skill and judgment of individual examiners, and (b) in any such series there will occur a considerable number of cases in which the proper diagnosis is doubtful.

Since the object of the epidemiologist is to define relative prevalence (or risk) by contrast in two populations, the primary requisite of an epidemiologic method is comparability of results. With the present scoring method (as in dental caries), this comparability has been achieved through rigid suppression of examiner judgment, in favor of a disciplined application of criteria which

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accept only positive signs as an indication of disease. This limits reporting to relatively late stages, wherein the signs are clear, obvious, and unmistakable. In consequence, population scores so obtained represent an underestimation from the clinical point of view, but the degree of underestimation is uniform throughout the study groups, and its magnitude can be computed. This is the usual situation when an epidemiologic measure is applied and is unavoidable if comparability is to be attained.

Like the DMF examination, the basic method is capable of the many permutations which follow the application of special examination skills and equipment, and might prove useful in a clinical study situation. With the exception of the brief test to be described, however, that possibility has not been explored here.

The method is essentially similar to that followed in scoring dental caries in experimental animals,[16] or in assessing dental fluorosis in human populations.[17]

The condition of the investing tissues is estimated individually for each tooth in the mouth, and is scored according to a progressive scale which gives relatively little weight to soft tissue inflammation and relatively great weight to destruction of bone. The score for an individual patient is the arithmetic average of the scores for the teeth in his mouth. The population score is the arithmetic average of the individual scores for the persons examined. Scores may be computed for a population of teeth (for example, for mandibular first incisors) as well as for persons.

Required field equipment has been reduced to a minimum. Mouth mirror and explorer are supplemented occasionally by a straight Jacquette scaler or the chip blower for demonstration of a periodontal pocket. Adequate light is essential, preferably from a source corrected to the approximate color of sunlight.

Criteria for field scoring are outlined in Table I, which also summarizes the x-ray criteria used in the clinical test of the method, to be described later in this paper.

Each tooth is scored according to the clinical condition of its supporting tissues. In the absence of overt inflammation in the free gingivae, or loss of function due to destruction of investing tissues, a tooth is considered to be negative and is assigned a score of zero. The scale provides for two stages of simple gingivitis, differing only in the extent of inflammation, and scored 1 and 2. A score of 6 is assigned when a periodontal pocket is demonstrated, except when tissue destruction is so far advanced that masticatory function has been impaired; in this case the highest score, 8, is given. When radiographs are studied it is sometimes possible to detect bone resorption prior to the appearance of a frank periodontal pocket. In the clinical test such cases were assigned an intermediate score of 4.

Several weighting systems were tried and discarded before the present scoring scale was adopted. It is designed to place the greater emphasis on advanced disease, and to minimize examiner disagreement. In application, the most difficult decision required of the examiner would seem to be that of distinguishing between negative gingivae and gingivae with mild gingivitis.

TABLE I

CRITERIA FOR THE PERIODONTAL SCORE

SCORE	CRITERIA AND SCORING FOR FIELD STUDIES	ADDITIONAL X-RAY CRITERIA FOLLOWED IN THE CLINICAL TEST
0	*Negative.* There is neither overt inflammation in the investing tissues nor loss of function due to destruction of supporting tissues	Radiographic appearance is essentially normal
1	*Mild Gingivitis.* There is an overt area of inflammation in the free gingivae, but this area does not circumscribe the tooth	
2	*Gingivitis.* Inflammation completely circumscribes the tooth, but there is no apparent break in the epithelial attachment	
4	(Not used in the field study)	There is early, notchlike resorption of the alveolar crest
6	*Gingivitis with Pocket Formation.* The epithelial attachment has been broken and there is a pocket (not merely a deepened gingival crevice due to swelling in the free gingivae). There is no interference with normal masticatory function, the tooth is firm in its socket, and has not drifted	There is horizontal bone loss involving the entire alveolar crest, up to half of the length of the tooth root (distance from apex to cemento-enamel junction)
8	*Advanced Destruction with Loss of Masticatory Function.* The tooth may be loose; may have drifted; may sound dull on percussion with a metallic instrument; may be depressible in its socket	There is advanced bone loss, involving more than one-half of the length of the tooth root; or a definite intrabony pocket with definite widening of the periodontal membrane. There may be root resorption, or rarefaction at the apex
RULE: When in doubt, assign the lesser score		

Comparability at this point is enhanced if a score for gingivitis is assigned only when the inflammation is clearly evident at first glance in good light and the effect of disagreement here is minimized by the manner in which scoring is weighted. Throughout the scale, the problem of the questionable diagnosis is avoided by the rule which instructs the examiner to assign the lesser score whenever he finds himself in doubt.

The score reflects the average status of periodontal tissues in a given mouth, without reference to the type or etiology of disease. In our studies the latter information has been recorded on the examination form as a footnote.

THE CLINICAL TEST

The effect of field underestimations upon group scores was tested in what was, essentially, a clinical study.* It dealt with 200 consecutive patients in the Clinical Center of the National Institutes of Health who were given both visual and x-ray examinations in the Dental Clinic, beginning in December of 1953. Some had been referred to the National Institute of Dental Research as possible

*For example, one in which the observer is free to use his highest skill and judgment, and auxiliary diagnostic aids, in the interest of complete reporting and at the sacrifice of quantitative comparability with a similar study carried out by a different observer.

participants in a study of methods used in periodontal treatment. Most of them had been admitted for study of a systemic ailment by one of the other Institutes.

All of the examinations followed the same routine. The periodontal score was the first item recorded. A paraffin-stimulated sample of saliva was then taken for bacteriologic study. This procedure was followed by a detailed oral examination, using mouth mirror and explorer. Carious lesions, missing teeth, and restorations were sketched on a representational tooth chart. The condition of the head and neck, temporomandibular joint, lips, labial and buccal mucosa, sublingual area, tongue, pharynx and tonsillar region, the hard palate, and the state of occlusion were specifically observed and described. Fourteen periapical x-ray films and two posterior bite-wing films were then exposed. Final diagnoses were dictated by the examiner after study of the patient's clinical records and of the dried and mounted x-ray films, with the aid of notes made at the time of examination which described the color, texture, tone, and general status of the gingival tissues.

All of the examinations were carried out by regularly assigned dental clinic personnel, with the exception of a sample of periodontal examinations performed by the author for purposes of calibration. In each case the x-ray films were studied, and scores revised, according to the criteria listed in Table I, by the author without reference to the examiner's final findings.

The effect of this procedure was to raise the mean score for the group as a whole from 1.20 to 1.45. There was a general upward revision at all levels of disease, as shown in detail in Table II, with a high degree of correlation between the scores derived by the two methods ($r = +.83$). The relationship was approximately straight line, as shown in Fig. 1.

TABLE II

THE RELATION BETWEEN PERIODONTAL SCORES ASSESSED BY VISUAL EXAMINATION ALONE, AND BY VISUAL EXAMINATION SUPPLEMENTED BY X-RAY, IN 200 CONSECUTIVE PATIENTS SEEN IN THE DENTAL CLINIC OF THE CLINICAL CENTER AT THE NATIONAL INSTITUTES OF HEALTH, BETHESDA, MARYLAND

RANGE OF PERIODONTAL SCORES, VISUAL ALONE	NUMBER OF PERSONS EXAMINED	MEAN SCORES	
		VISUAL ALONE	VISUAL, SUPPLEMENTED BY X-RAY
zero	77	zero	.17
.1- .3	34	.20	.42
.4- .9	26	.63	.83
1.0-1.9	23	1.33	1.97
2.0-4.9	24	3.41	3.77
5.0-8.0	16	6.54	6.60
All persons examined	200	1.20	1.45

In 128 of the 200 cases there was no difference between the original score (applied as it would have been used in the field) and the final score after study of the radiographs. In 59 additional cases, the average underestimation was 0.4 per person, with nearly all of the difference due to the discovery of

beginning bone resorption, usually associated with a proximal restoration. In the remaining 13 cases, the severity of disease was grossly underestimated by the field method, with x-ray revision raising the average scores per person by 1.9.

While this finding presents an excellent argument that the x-ray is essential in patient treatment, or in the usual clinical study where individual patient response is important, such deviations are quite tolerable in the epidemiologic study so long as they are random, i.e., as apt to occur in one group as in another. These were research study patients, and the available data concerning their physical condition and social background were exceptionally detailed and complete. No association could be discovered between scoring differences, as returned by the 2 methods of examination, and age, sex, race, or the general sort or specific type of physical ailment. It was concluded that the field method will return a gross underestimation in from 3 to 10 per cent of individual cases when groups of this size are studied, and that these cases will be randomly distributed throughout the study populations.

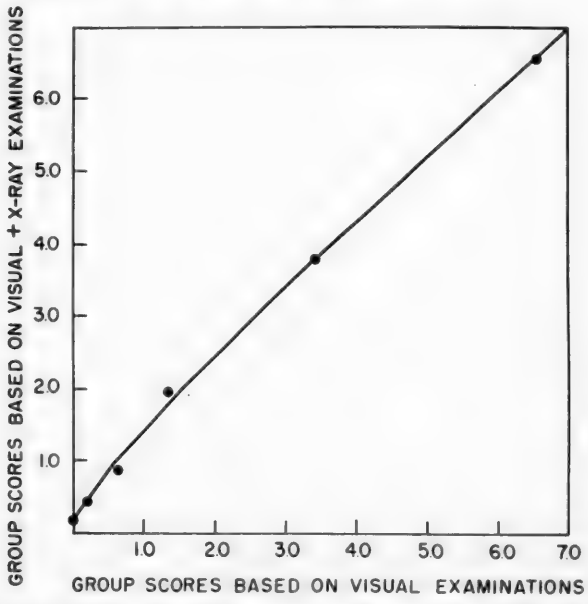

Fig. 1.—Visual and visual plus x-ray periodontal scores in 200 Clinical Center patients.

Given these conditions, and so far as the difference between 2 groups is concerned, it is obvious that about the same finding will be returned whether x-ray is used, or whether the estimate is based upon the field examination alone.

By way of illustration, in this study group white males show slightly but not significantly higher mean scores than do white females, by either method of appraisal. Using the field method the respective mean scores are 1.27 and 1.04 with a P value of .46*: using the clinical method, the respective scores are 1.55 and 1.30, with a P value of .45.

*A difference as great or greater, due to sampling error alone, may be expected in about 46 of 100 similar trials.

The same order of comparability between these methods was observed throughout a series of experimental sortings, in which the 200 examination cards were allocated to artificial study groups with predetermined characteristics. A sort designed to produce approximately equal scores in 2 equal divisions yielded scores of 1.18 against 1.24 with the field method of examination, contrasted with 1.44 against 1.48 with the clinical method, with P values of .83 and .89, respectively. A sort based upon a clinically significant difference (all cases in which extensive extractions were required because of periodontal disease were assigned to the same group, with other differences balanced) yielded field scores of .79 vs. 1.61, and clinical scores of .96 vs. 1.94, with a P value of less than .01 in both cases. A sort for extreme differences produced field scores of .11 vs. 2.31, and clinical scores of .25 vs. 2.67; in both instances the difference between the 2 scores was about 8 times its standard error.

In an independent study, the field score was applied by Examiner E to 110 long-term residents of Bartlett, and Cameron, Texas. Fourteen periapical x-ray films were then exposed for each patient. These films were studied independently by 4 dental examiners who assessed the amount of alveolar bone resorption.[18] Mean periodontal scores for persons with no bone resorption, or with mild, moderate, or severe bone resorption, were as follows:

BONE RESORPTION ON X-RAY (CONSENSUS OF FOUR EXAMINERS)	NUMBER OF PERSONS	MEAN PERIODONTAL SCORE BY FIELD METHOD
None	11	.89
Mild	51	1.14
Moderate	34	2.13
Severe	14	4.81

On the basis of these 2 tests it was concluded that the field method of scoring is adequate to determine the relative periodontal status of 2 groups or populations.

RELATION OF SCORES TO CLINICAL SEVERITY

A numerical score is useful in epidemiologic studies only if it corresponds, in magnitude, with the severity of the disease to which it is applied. To illustrate the relationship between the present score and periodontal disease, the findings for persons in the Clinical Center series were tabulated according to the final diagnosis, arrived at by the individual examiner after his careful study of the patient's x-ray films and clinical records.

Despite some confusion in terminology, it was possible to determine from his summary of the case whether the examiner had considered the periodontal tissues to be essentially normal in a given patient, or whether there was a gingivitis without appreciable bone loss, or whether the patient was in a state of transition from simple gingivitis to more severe tissue destruction, or whether periodontal disease (designated as "periodontoclasia" on the examination form) was frank and established, or whether it had progressed to a terminal stage (as evidenced by his recommendation for extensive extractions). On these bases the patients were divided into 5 groups with increasingly severe

disease, and mean scores and standard deviations were computed for each group (Table III). About the same patterns were seen when results were tabulated separately for the several examiners, except that there was true disagreement among them as to the dividing line between simple gingivitis and incipient "periodontoclasia."

TABLE III

RELATIONS BETWEEN CLINICAL DIAGNOSES, MEAN PERIODONTAL SCORES, AND SCORING RANGES IN TWO GROUPS OF ADULTS

	200 CLINICAL CENTER PATIENTS, 1953-54			601 COLORADO ADULTS, 1950		
	EXAMINERS: A, B, C, D			EXAMINER: A		
CLINICAL DIAGNOSES	NUMBER EXAMINED	MEAN SCORE	MOST SCORES IN RANGE OF*	NUMBER EXAMINED	MEAN SCORE	MOST SCORES IN RANGE OF*
Normal	79	.01	0 to .1	347	.04	.0 to .2
Gingivitis	49	.53	.1 to 1.0	116	.59	.3 to .9
Severe gingivitis to incipient periodontoclasia	16	.77	.3 to 1.3	77	1.31	.7 to 1.9
Periodontoclasia	44	2.81	1.0 to 4.6	51	3.29	1.6 to 5.0
Terminal periodontoclasia	12	6.43	4.9 to 8.0	10	5.94	3.8 to 8.0
All cases	200	1.20	0 to 3.2	601	.68	.0 to 1.9

*Mean score plus and minus one standard deviation of the mean.

Table III also includes the array of means and standard deviation intervals as determined for the first adult group to which the field scoring method was applied in its present form. This group included 601 Colorado natives aged 16 through 44 years. They were examined in 1950.[19]

EXAMINER COMPARABILITY

In cases of gingivitis, or more advanced disease, examiner agreement is excellent, given examiners who are well calibrated and who are so familiar with the criteria that their responses are very nearly automatic acts. When frank disease was encountered there was virtually no difference between the 3 principal examiners in the Clinical Center series, as judged by calibration with examiner A, or by the deviations of their field-method scores from x-ray findings for the same persons. Most disagreement occurs at the threshold point, i.e., at the point of deciding whether a given area of free gingiva is normal or slightly inflamed. Here examiners may differ systematically, despite efforts at calibration. This has little effect on group averages in most adult populations, where the means tend to be dominated by high-scoring individuals with advanced disease. In young populations, where mild gingivitis is usually the most severe stage of disease to be seen and the majority of individuals score zero, the effect of such disagreement is more overt. Some typical findings returned by 4 trained and calibrated epidemiologic examiners, for children in whom the general level of disease was low, are listed in Table IV. Where dual examiners are indicated in this tabulation, the 2 men saw equivalent samples from the same universe and, within the limits of sampling error, should have returned the same estimates. It will be observed that examiner G is consistently higher than his fellows by a margin of about .05, but that examiners

A, E, and F tend to report similar scores. It may be prudent to consider differences of such small magnitude to be meaningless, particularly when group scores fall within the limits of "normal" as estimated in Table III (i.e., from zero to .1 or .2) unless both groups have been surveyed by the same examiner.

TABLE IV

PERIODONTAL SCORES RETURNED BY VARIOUS EXAMINERS FOR VARIOUS POPULATIONS OF CHILDREN

POPULATION	NUMBER EXAMINED	MEAN AGE (YEARS)	EXAMINER (S)	PERIODONTAL SCORE
a. Boys and girls aged 12 to 14 years in a Junior High School, Maryland, 1952	286	13.1	A	.03
	244	13.1	E	.03
b. Boys and girls aged 12 to 14 years in a Junior High School (adjacent to school *a*), Maryland, 1952	359	13.0	F	.06
c. Boys and girls aged 12 to 14 years in a Junior High School, Maryland (same school as in *a*), 1954	270	13.0	A	.07
	285	13.0	G	.11
d. Boys and girls aged 12 to 14 years in a Junior High School, Maryland, 1952	234	13.0	A	.05
	236	13.1	E	.05
e. Boys and girls aged 12 to 14 years in a Junior High School, Maryland (same school as in *d*), 1953	117	13.7	E	.14
	198	13.8	G	.20
f. Eleventh grade boys and girls in Michigan, 1952	73	15.9	A	.08
	80	15.9	F	.05
g. Eleventh grade boys and girls in Michigan, 1952	73	16.2	A	.08
	75	16.0	F	.08
h. Boys and girls aged 12 to 14 years in Illinois, 1953	585	13.0	E	.09

DISCUSSION

In the evolution of this scoring method to its present form, several devices have been tested and discarded, in the interest of comparability or to eliminate needless effort.

For purposes of group scoring, for example, information on actual pocket depth (either linearly, as in millimeters or anatomically, in terms of bifurcation or trifurcation involvement) added little and proved to be a troublesome focus of examiner disagreement. Observers were more readily able to agree whether a pocket was present or not and, if present, whether or not function had been impaired. This differentiation proved fine enough to provide delicate gradations in scoring.

Histories of missing teeth were found to be redundant. If an individual has already lost teeth because of periodontal involvement, there is a strong likelihood that his remaining teeth will show extensive disease.

The progressive loss of teeth with advancing disease requires that the score be expressed as the average condition of the teeth remaining in the mouth. A score expressed as a total number of units may drop sharply, in population groups, as disease advances and teeth are lost.

Scores based upon the anterior teeth alone, though adequate to define relative prevalence in populations with no more than mild gingivitis, failed to disclose real differences in groups of persons with more advanced periodontal disease.

Individual scores should be rounded to not more than 1 decimal place and population scores to not more than 2 decimal places.

Tabulation from coded examination cards requires about the same clerical effort as the summation of any single item of dental caries information (such as the number of decayed but unfilled teeth). Scores for the 585 children listed under (*h*) in Table IV were computed and tabulated as frequency distributions in about 3 hours' clerical time.

While these distributions tend to show pronounced right skewness, normal constants are ordinarily appropriate.

Unlike the number of decayed, missing, or filled permanent teeth, which is the cumulative measure of the patient's lifetime caries experience, the periodontal score is a morbidity index, and may regress following treatment.

SUMMARY

This report describes a quantitative method of scoring periodontal disease for field population studies. The method requires a minimum of equipment and returns comparable results. Its criteria are described and its rationale is discussed. The basic method may have value, with appropriate modifications, in the clinical study.

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USE OF EPOXY RESINS IN RESTORATIVE MATERIALS

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INTRODUCTION

MODERN chemistry has developed a large number of synthetic resins. Some of the new types are exceptionally heat resistant, others chemically inert, and others very adhesive to certain materials. Not all of these have been examined extensively with an object of finding an application in dentistry. It is conceivable that some of these, in addition to the methyl methacrylate resin, may find considerable use in dentistry.

The methacrylate resin introduced to the dental profession as early as 1937,[1] has been the one most carefully studied and most commonly used by the dental profession. The properties of polymethyl methacrylate have been carefully described by Paffenbarger, Nelsen, Sweeney, and Coy.[2, 3] Their properties included certain limitations—a coefficient of thermal expansion that is not equivalent to that of tooth structure, less than optimum adhesion to tooth structure, and lack of color stability for the direct filling resins.

The silicate cements have the well-known limitation of relative solubility and disintegration in the oral environment.[4]

These limitations led to the study and consideration of synthetic resins other than methyl methacrylate for use as a dental filling material. The use of many of the currently available resins is precluded by their color, curing temperature, or inability to take inert fillers.[5] However, one of the relatively new types, the epoxy resin, appeared to be worth investigation. The purpose of this report is to describe gross experimental studies of one epoxy resin.[6] This investigation was primarily aimed at developing a dental material with the following combined properties: a coefficient of thermal expansion equivalent to that of tooth structure, adhesion to the tooth, color stability, and insolubility in the mouth.

MATERIALS AND METHODS

Coefficient of Thermal Expansion.—The tooth expands or contracts across the crown approximately 11 ppm per degree centigrade.[2] Without any filler, one epoxy resin[6] has a linear dimensional change of about 67 ppm per degree centigrade. However, if a filler with a low coefficient of thermal expansion is added, the resultant mixture will have a lower thermal expansion. The outstanding adhesive quality of the epoxy resins suggests their use as a binding agent for materials with low coefficient of thermal expansion. Fused quartz, for

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example, has a linear change of 0.2 to 0.6 ppm per degree centigrade,[7] and may be used as a filler for this resin.[6] Fused quartz powder is the fundamental filler used in the mixtures studied in this investigation, where the material is used for tooth restorations. Other fillers are preferable when the material is used for denture bases or other purposes.

Fused quartz powder, produced by crushing, was sifted through silk screens of known mesh. Particles finer than 325 mesh were separated by an air column. The average diameters of the particles of the separated portions were estimated by study under optical magnification. Measured amounts of certain of the portions were reunited, duplicating as nearly as possible the relationships indicated in Table I.

TABLE I

PARTICLES	AVERAGE DIAMETER OF PARTICLES (IN ARBITRARY UNITS)	COMPARATIVE NUMBER OF PARTICLES
P_1	1	1
P_2	8^{-1}	46
P_3	8^{-2}	46^2

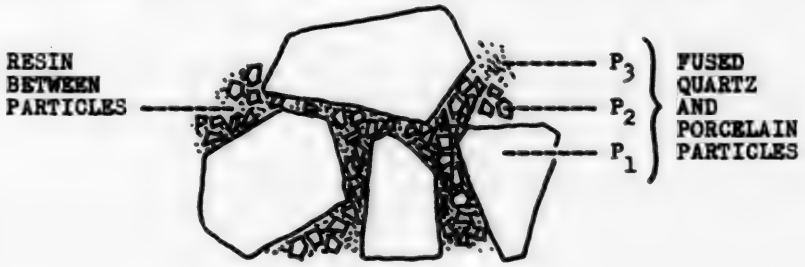


Fig. 1.

Mixtures produced in this way appeared to have good strength, and tests indicated that thermal expansion as low as 10 ppm per degree centigrade and lower was obtained. The filler to resin ratio of some of the mixtures was greater than 4 to 1. The coefficient of thermal expansion of 22 samples is indicated in Table II.

Adhesion to Tooth Structure.—Tests were conducted to determine the adhesive strength of the mixtures described above to the prepared tooth surface. Molar teeth were embedded in dental stone. The occlusal surface was ground away with a cast trimmer until a flat unbroken surface was obtained. Epoxy mixtures were allowed to cure in contact with this surface and an arm of a testing device. The materials were placed in contact but no pressure was applied.

Adhesion was tested by pulling the mixture from the surface in a direction parallel to the surface, producing a shear disruption, or perpendicular to the surface, producing a tensile disruption. A spring scale and levers were used to measure the force required for disruption.

The force required for separation varied with the time and temperature of curing, condition of surface when mixture was applied, and temperature and exposure to separating fluids when adhesion was tested. The results of these tests are given in Table III.

TABLE II

SAMPLE TESTED	LINEAR COEFFICIENT OF THERMAL EXPANSION PER DEGREE CENTIGRADE X 10^{-6}
F—1	66
F—2	65
F—7	61
F—8	61
F—24	58
F—11	49
F—6	46
F—10	45
F—22	43
F—25	38
F—13	32
F—12	31
F—14	27
F—15	22
F—19	20
F—20	18
F—19A	17
F—21	14
Teeth across crowns*	12
Teeth across crowns*	11
F—23	10
F—28	8.0
F—27	6.3
F—26	5.3

*According to same device with which samples measured. These samples represent experimental formulations of epoxy resins with various fillers and ingredients.

Color Stability.—To test color stability during exposure to ultraviolet rays and light, a sample of the mixture was divided, and one half was exposed to a Westinghouse 275 W Sun Lamp at a distance of 4 feet, 15 minutes per day, for 4 months. The other portion was kept in the dark. When the 2 were put side by side and compared, there was no difference in shade or color visible to the unaided eye.

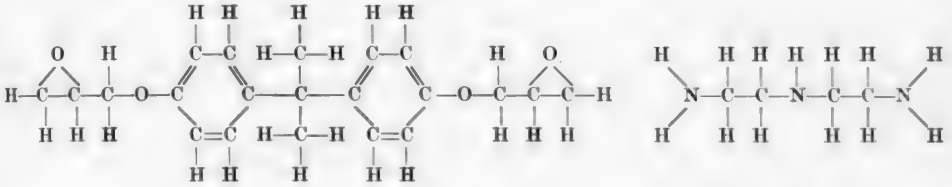
In a test conducted by the manufacturer, an uncured epoxy resin[8] was exposed to ultraviolet radiation continuously. After exposure for 3 months, the resin was still a clear transparent amber, but the color had approximately twice the original intensity. Beyond 3 months, the resin began to bleach and the color intensity decreased.

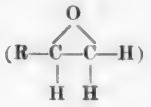
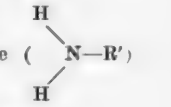
When a filler to resin ratio such as is described for this mixture is used, the relatively small intrinsic color change of the resin probably produces no appreciable color change of the total mixture. If further tests prove it necessary, the resin could be radiated before the mixture is prepared and pigments are added. Other sources of color change will be considered in the discussion.

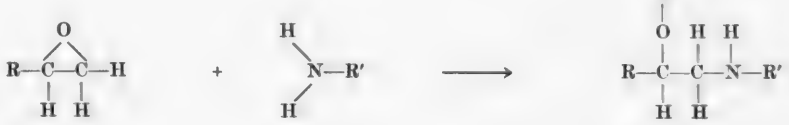
DISCUSSION

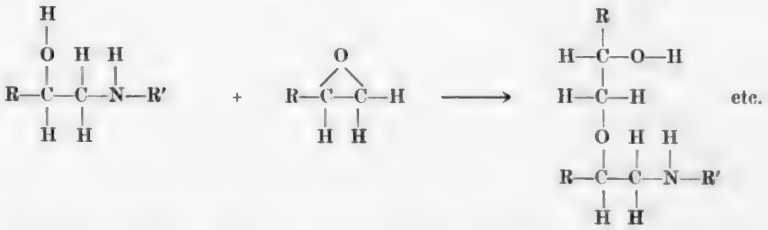
Epoxy Resins.—The relatively new epoxy resins were developed about 1940 and have found uses of commercial importance only in the last 2 or 3 years.[8] Epoxy resins are outstanding in adhesion to various materials such as metals, wood, plastics, and glass.[6] They are thermosetting resins that will cure at room temperature. They form solids having good strength and chemical stability. They have a transparent, light amber or straw color that allows al-

most unlimited color possibilities when appropriate pigments are used. In the uncured state at room temperature, one epoxy resin[6] is a viscous liquid. After the curing agent is mixed with it, the resin becomes increasingly viscous and cures to an infusible solid. The high degree of cross linkage between polymer chains is believed to give the infusible thermoset properties. A thermoset resin, such as a cured epoxy resin, is generally less affected by solvents, chemicals, and heat than a thermoplastic resin such as methyl methacrylate. The polymerizing process occurs through the epoxide groups, the three-membered rings containing oxygen. In the epoxy group, the ring exists in a strained and somewhat unstable condition and is very prone to open and combine with compounds having an available hydrogen. Reaction (1) is spontaneous at room temperature.[9] Under proper conditions, other epoxy groups then react with the newly formed hydroxy radicals, forming the cross linkage that gives the thermoset characteristics to the cured resin, reaction (2).



An epoxy resin[6, 8]  Diethylenetriamine 

(1) 

(2) 

The reactions are exothermic and irreversible. The relatively small amount of resin and bulk of inert filler would probably preclude any overheating of a tooth by the reaction in vivo.

Coefficient of Thermal Expansion.—To obtain a coefficient of thermal expansion as low as tooth structure, a high filler to resin ratio was found necessary. To obtain this, while maintaining strength of the cured mixture, special attention was given to the relative sizes of the filler particles. These sizes were

calculated so that smaller particles would fit in the spaces between tightly packed larger particles (Fig. 1). In effect, these smaller particles replaced their volume of resin when the resin was added to produce the mixture. It was reasoned that so long as there were no voids or filler-particle surfaces that were not wetted by the resin, raising the filler to resin ratio, per se, would not greatly weaken the mixture (Table I, Fig. 1).

Adhesion to the Tooth.—While the epoxy type of resin has outstanding adhesion to metals, wood, glass, and most plastics, there is little or no bonding to polyethylene, fluorinated thermoplastic polymers, cellulose acetate, carrauba wax, and certain silicones.[6] The prepared tooth surface, in vivo, is not a simple material that would allow the degree of adhesion of an epoxy resin to be predicted.[10]

TABLE III

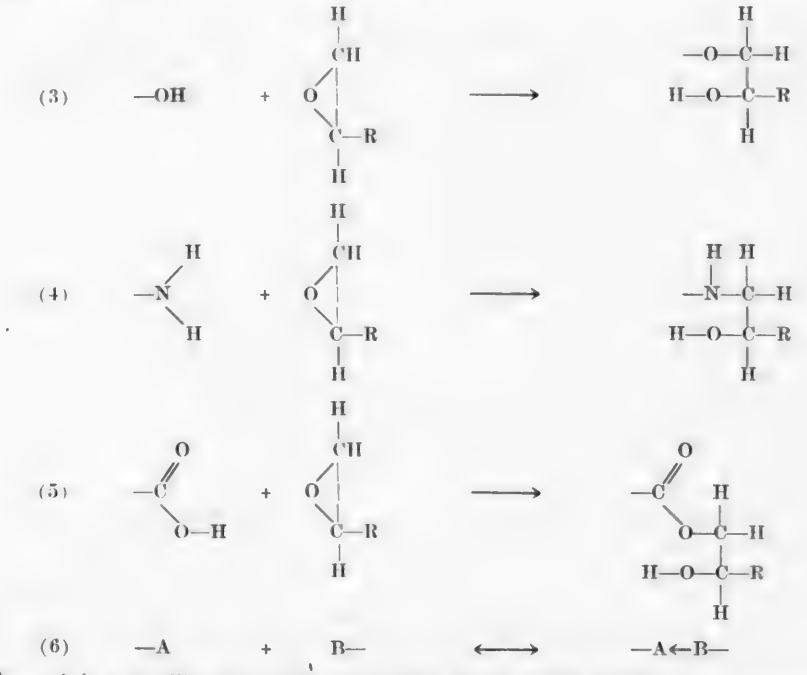
SAMPLE, OR FORMULATION NUMBER	APPROXIMATE ADHESION IN LB. PER SQ. IN.	CURING TIME AND TEMPERATURE IN ° F.	TOOTH SURFACE WHEN APPLIED	WETTING SOLUTIONS USED	ADHESION TEST. CONDITIONS
F 24	300 shear; delamination	48 hours; room temp.	Dry, untreated	H_2O and detergent 24 hours	Wet, room temperature
F 24A	210 shear	30 minutes; 240° F.	Dry, untreated	None	Dry, room temperature
F 25	160 shear	Room temp.; poor mixture; stayed soft	Dry, untreated	H_2O and detergent 24 hours	Wet, room temperature
F 26	400 shear; delamination	Room temp.	ATA Solution 30 minutes; acetone, Res. layer, I. R. 10 minutes	H_2O and detergent 30 min.	Wet, room temperature
F 27	470 tensile; delamination	Three weeks; room temp.	Dry, untreated	H_2O and detergent two weeks, 10% alcohol	Wet, room temperature
F 28	None	Two weeks; room temp.	Dry, untreated	H_2O and detergent, 10% acetone; Immersed immediately after appl.	Wet, room temperature
F 29	740 shear; delamination	Two weeks; room temp.	EDTA Solution one minute acetone-washed	H_2O two days	Wet, room temperature

However, the surface of glass and wood under atmospheric conditions consists essentially of exposed –OH groups.[11] The cured epoxy resin also contains many free –OH groups.[8] One theory of the adhesion in such cases is that of electrostatic attraction between materials of similar polarity.[8] The positive surface charge of the apatite crystal, which makes up a large portion of the tooth, can be raised or lowered by heterionic exchange.[10] The treatment of the cut surface of the tooth with ethylene diamine tetra-acetic acid probably re-

places the available calcium ions that it sequesters or chelates from the tooth, with hydrogen ions (hydronium ions, H_3O^+) which it gives up. The resulting change in the electrostatic charge of the tooth surface may then approximate that of other materials with surfaces consisting essentially of exposed –OH groups.

To remove loosely bound calcium ions and to adjust the polarity, the cut surface of the tooth was washed with a solution of ammonium triacetic acid or ethylenediamine tetra-acetic acid which are chelating compounds that actively sequester metallic ions. These treatments empirically improved the strength and moisture resistance of the resin's adhesive bond (Table III).

While the adhesion of this type of resin to the surfaces of most materials is considered to be due only to electrostatic or ionic adhesive forces,[8] it would appear possible that coordinate or covalent bonding might occur between the epoxy type resin and the prepared tooth surface under the proper conditions. When the exposed protein phase of the tooth is included, the essentially free groups and their possible reactions are as follows:



Where: A is a metallic atom (such as a calcium of the apatite lattice),
B is an electron donor (such as a nitrogen or oxygen atom of the resin molecule) and
← represents a coordinate bond.

Since reaction (5) would give a product with relatively low water stability, an attempt was made to remove carboxyl radicals from the free surface of the tooth. Various decarboxylating agents were used. While these reactions are hypothetical, it should be noted that the epoxy group is a very reactive one and

that in some of the tests there was delamination of the tooth or mixture when adhesion was tested, even after 24 hours of submersion in wetting solutions (Table III). Whatever the mechanism of bonding, the attachment or adhesion is increased by the fact that the coefficient of thermal expansion of both the mixture and the tooth are nearly equal, and by the mixture having relatively little shrinkage during cure.[11]

Color Stability.—While no conclusive testing to determine color stability has been made on this epoxy mixture, certain comparisons with the self-curing methyl methacrylate resins are encouraging. The epoxy mixture cures at room temperature without promoters or catalysts which tend to discolor the methacrylate resins.[2] The high proportion of inert filler in the epoxy mixture decreases the over-all effect from light and ultraviolet radiations upon the cured mixture. If the coefficient of thermal expansion is equivalent to the tooth, there is good bonding or adhesion to the walls of the cavity and there is little curing shrinkage, there should be little or no seepage or percolation of material between the restoration and the tooth surfaces such as occurs with the methacrylate resins.[2] Since the curing of the epoxy mixture is by addition and no oxygen-releasing catalysts are required, there should be no porosity from curing products to allow infiltration of debris.

Other Properties Observed.—Tests conducted on samples of this mixture indicated the following approximate values:

X-ray transmission of the mixture is very nearly equal to the same thickness of normal tooth dentin. The inclusion of a small amount of strontium phosphate or sulfate, barium sulfate, zirconium oxide or other compound containing one of the heavier elements imparts radiopacity to the mixture. Thus x-ray transmission may be controlled since such fillers are compatible with epoxy resins.

The mixture has no taste when cured. The resin has a slight, but not objectionable odor, which disappears soon after the mixture is cured.

After immersion in 52 per cent hydrofluoric acid for 30 minutes, only 0.2 mm. of surface was softened; deeper, it appeared unchanged.

Samples were immersed in 50 per cent ethyl alcohol for 2 weeks with no gross or visible damage. When pure acetone was used, 2 weeks' immersion caused noticeable swelling and weakening of the samples.

Abrasion resistance at normal temperature was greater than tooth enamel or dentin for the samples tested in vitro. This was compared by measuring the depth removed due to dragging the specimen across garnet paper wetted with kerosene, with equal pressure. In vivo, however, an occlusal inlay in a lower first molar showed greater attrition than the surrounding enamel after 1 year of service. The resistance to abrasion varies with the hardness of the filler particles, with the ratio of particle size of filler to particle size of the abrading surface, and inversely with the temperature. For example, if the material was filled with large particles of an abrasive such as aluminum or zirconium oxide,

it would be highly abrasive at normal temperatures. However, if finely powdered talc were used as a filler, it would be easily abraded. When finely divided porcelain and fused quartz are used as fillers, abrasion resistance is near that of tooth structure.

Crushing strength ranged from 1,400 to 2,540 pounds per square inch for the samples tested.

The shrinkage during cure of one pure epoxy resin is given as, "About 2 per cent before gelation and about 2 per cent after gelation. This can be reduced by the addition of fillers such as glass, asbestos, silica, and alumina."[6] With the filler to resin ratio used it is estimated that shrinkage during cure would be about 0.6 per cent before gelation and less than 0.5 per cent after gelation.

A small amount of anhydrous calcium oxide incorporated in 2 samples appeared to produce expansion during cure. This may have been due to hygroscopic action of CaO or reaction of CaO with –OH groups. Shrinkage during cure might be controlled by some such ingredient. Since the polymerization of the epoxy resin is by addition and no volatile products are released, the amount of intrinsic shrinkage during cure is relatively small.

The coefficient of thermal conductivity on one pure epoxy resin[6] is 0.00048 cal/sec/cm²/deg C/cm. It is estimated that the mixture used in this study would have a coefficient of thermal conductivity of about 0.002 cal/sec/cm²/deg C/cm. This would be considerably nearer the thermal conductivity of the tooth than metallic filling materials.

Penicillin, Aureomycin, and Terramycin were added to separate portions of epoxy resin and samples cured. These were placed upon nutrient agar plates freshly innoculated with *Esch. coli*. There were zones of inhibition around samples containing the antibiotics.[12]

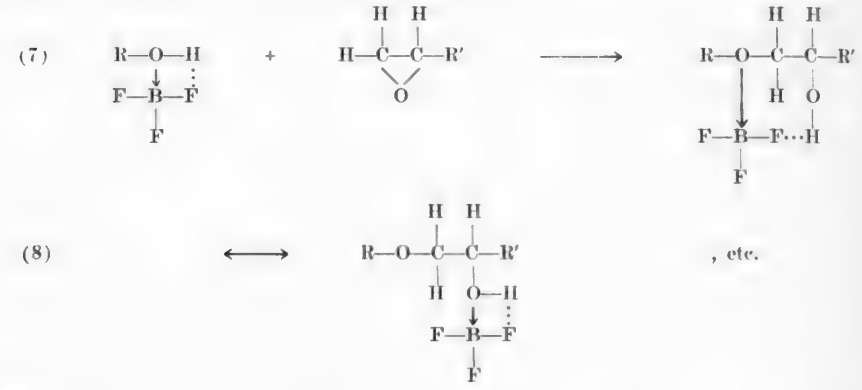
The water absorption for one epoxy resin is 0.07 per cent in 24 hours, 0.20 per cent in 1 week, and 0.47 per cent in 1 month.[6] The water absorption for the mixture would be influenced by the amount and type of fillers and the type of curing agent used.

This resin is relatively stable to chemicals. The percentage gain in weight after 1 month's immersion in ethyl alcohol is 0.75 per cent and in benzene is 0.26 per cent.[6] The percentage gain for the mixture should be less in proportion to the amount of inert filler.

The setting or hardening time can be varied between 1 minute and many hours depending on the type of curing agent, filler to resin ratio, catalysts, and other factors.

There are a number of agents that will accelerate or catalyze the polymerization of the epoxy resins. There are sulfonic acid catalysts, such as p-toluene sulfonic acid, but these produce ester bonds within the resin, giving the product less water stability.[8] Polybasic acids such as phosphoric acid will give rapid polymerization at room temperature.

Boron trifluoride[13] and stannic chloride[8] are catalysts which produce rapid polymerization of epoxy resins. Reactions (7) and (8) are suggested as a possible mechanism of the polymerization by BF_3:



(8)

, etc.

Where: ← represents a coordinate bond,
. . . represents a hydrogen bond and the H of a –OH group is made more labile by the coordination of the BF_3 group.

In this reaction, ether bonds are formed which should give a product with good water stability and chemical inertness. Toxicity studies would have to be made on these or other catalysts before their usefulness in a dental filling mixture could be determined. Amine curing agents and acid-acting catalysts do not appear compatible for room temperature curing of epoxy resins.

The toxicity of the uncured epoxy resins is relatively low. The oral administration of more than 7 c.c. of one of the epoxy resins per kilogram of body weight was tolerated by white mice without symptoms. There is no hazard from vapor exposure.[14] Repeated contact with uncured resin might produce skin irritation in hypersensitive individuals, and skin contact should be avoided. After polymerization or curing, the resin is nontoxic and inert. However, the undiluted amine curing agents are caustic and produce dermatitis by contact. They are strongly basic compounds, and their toxicity is mainly a local action due to their strong alkalinity. If adequate concentrations of their vapors are inhaled, there is irritation of the mucous membranes. Vapor concentrations attainable at ordinary temperatures would not produce systemic poisoning, as they are not particularly toxic systemically.[15] The amounts required as a curing agent for these dental mixtures are small. The curing agent may be reacted with a certain amount of the resin and diluted with a portion of the filler prior to its use to reduce its irritating effect should skin contact occur as it is being added to the resin mixture. Since only about 8 parts of the amine curing agent are added to 100 parts of resin, the reaction between the 2 commences immediately, and they are diluted by a greater volume of inert filler, irritating properties or toxicity of the soft and pliable mixture should

be no problem. Allergic manifestations might occur, especially after repeated contact, but this should be no more common than with methyl methacrylate or other resins.

Study is now being made of other curing agents and catalysts, which are relatively nontoxic. Certain liquid polyamides and polyamines will cure epoxy resins, and some of the tetra-alkyl titanates are promising as adhesion promoters and perhaps reaction accelerators.

Although this report is of studies in vitro, clinical use of this material has commenced and the results thus far are encouraging.

SUMMARY

One of the relatively new synthetic resins now commercially available is the epoxy resin. This is a completely different type of resin from the methacrylate resin now being used in dentistry. The epoxy resins will cure at room temperature to produce a strong, chemically stable, thermoset solid with outstanding adhesiveness.

In this study, an epoxy resin is used as a binder for fine particles of fused silicon dioxide and porcelain.

With this mixture the following properties may be obtained in a dental filling material: (1) thermal expansion equal to that of tooth structure or 11 ppm per degree centigrade linear. (2) adhesive properties; (3) color stability; (4) apparent stability and insolubility in the oral environment.

Further investigation and development of this approach to dental restoration materials is indicated.

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MEASUREMENT OF THE REDUCING ACTIVITY OF HETERO- AND HOMOFERMENTATIVE LACTOBACILLI

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THE genus *Lactobacillus*, several of whose species are associated with the dental carious process, is classified into homo- and heterofermentative groups on the basis of the yield of lactic acid produced from the dissimilation of glucose.[1] Various strains of lactobacilli have also been found to have different reducing capacities. Gillespie and Rettger[2] reported that recently isolated oral lactobacilli established much more intense reduction potentials than less recently isolated nonoral strains of *Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus bifidus,* and Döderlein's bacillus. On the basis of such findings the authors postulated that a relationship may exist between the oxidation-reduction potential produced by a given species and its metabolic activity. Since differences may exist between the reducing ability of lactobacilli, these variations could be investigated advantageously through the use of suitable indicators which would act as hydrogen acceptors. An indicator of possible use in determining the reducing activity of lactobacilli is 2, 3, 5-triphenyltetrazolium chloride (TTC) which upon reduction changes from a colorless form to the red, water-insoluble compound, formazan. Studies made with TTC have established that living cells reduce it by enzymatic activity.[3] It was observed in previous unpublished studies that lactobacilli reacted differently in reducing TTC incorporated in several types of solid media upon which they were grown. An investigation was then made of the reduction of TTC by the homo- and heterofermentative lactobacilli, which is the basis for this report.

MATERIALS AND METHODS

Test Organisms.—Fifteen strains or species of lactobacilli employed in the succeeding experiments to determine their effect on TTC included the following: *Lactobacillus brevis* (Bb10), *Lactobacillus casei* (Da 318), *Lactobacillus acidophilus* (La 27) and *Lactobacillus fermenti* (Bl 28) obtained in the vegetative state from Dr. P. A. Hansen, University of Maryland; a lyophilized culture of *Lactobacillus delbrueckii* obtained from the Biologics Research Department, Army Medical Service Graduate School. The biochemical activity of these bacteria had been determined previously at the respective institutions from which they were obtained. Ten strains of lactobacilli isolated from human oral cavities and maintained in the lyophilized state were utilized also. The biochemical characteristics of these lactobacilli were determined earlier in con-

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nection with studies of their sensitivity to several antibiotics. Although they were not differentiated into species, they were divided into homo- or heterofermentative strains according to the following metabolic balance studies.

Metabolic Balance Studies.—The oral lactobacilli were subjected to metabolic balance studies to determine their homo- or heterofermentative characteristics by ascertaining the concentrations of lactic acid and glucose in Somogyi filtrates of cultures in brain heart infusion broth (Difco) by the methods of Barker and Summerson[4] and Schales and Schales,[5] respectively. Homofermentative lactobacilli produced 80 per cent or more lactic acid and heterofermentative lactobacilli produced less than 50 per cent lactic acid from the dissimilation of glucose.

Preparation of Cells for Determination of Reducing Activity.—The strains of lactobacilli were maintained on 3 per cent phytone-2 per cent dextrose agar. Prior to testing for reducing activity, each strain was transferred at least 3 times at 18-hour intervals in phytone-dextrose broth. To obtain the necessary quantity of cells, 1 ml. of the culture from the last transfer was inoculated into 100 ml. of the broth and incubated 18 hours at 37° C. The cells of the cultures were obtained by centrifuging at 2,000 r.p.m. (900 Gm.) for 30 minutes at 10 C. The sedimented cells were washed twice with cold 0.2 M phosphate buffer (pH 6.0) and resuspended in sufficient buffer solution to give a final nitrogen concentration of 0.8 to 1.0 mg. per ml. Such preparations of washed lactobacilli are hereafter referred to as the "standard test suspension."

Determination of Tetrazolium Reduction.—TTC reduction by the lactobacilli was determined by adding 1 ml. of M/50 glucose, and 0.22 ml. of 1 per cent TTC, each contained in 0.2 M phosphate buffer (pH 6.0), to the 1 ml. of the standard test suspension and incubating in a water bath for 1 hour at 45° C. All necessary precautions were taken to exclude light from influencing the reaction. The reduced TTC (red formazan) was extracted from the reaction mixture with acetone by the method of Kun and Abood.[6] The acetone extract was then measured in a Klett-Summerson colorimeter, utilizing a 420 filter. Colorimetric determinations of the acetone extracts, expressed as "Klett-Units" indicated relative estimates of TTC reduction. When color intensities of the solutions were too great to be determined with reasonable accuracy (Klett readings over 300), extracts were diluted 1:10 prior to making colorimetric readings and the resultant readings multiplied by 10.

EXPERIMENTAL RESULTS

In preliminary studies of the metabolic pattern of the 15 strains of lactobacilli, it was ascertained that *L. casei, L. acidophilus, L. delbrueckii,* L3, and L5 were homofermentative. *L. brevis, L. fermenti,* L4, L6, L7, L8, L9, L10, L11, and L12 were found to be heterofermentative.

The following factors were considered in determining conditions affecting the reduction of TTC by the lactobacilli: (1) final concentration of TTC, (2) age of test culture, (3) hydrogen-ion concentration, (4) temperature, (5) buffer molarity, and (6) cell concentration and endogenous reduction in the absence of glucose. With the exception of cell concentration, the influence of

the various factors upon TTC reduction differed only slightly between species and strains of lactobacilli; hence, the results are presented in each test only for 2 representative homofermentative and 2 representative heterofermentative types. To emphasize the consistent differences in reducing activity of the 2 metabolic types, data on the effect of variation of cell concentration on TTC reduction will be presented for all of the lactobacilli strains.

Tetrazolium Concentration.—The relationship of TTC concentration to the reducing activity of the lactobacilli was investigated by the addition of TTC, in concentrations ranging from 0.01 to 0.2 per cent, to equal volumes of diluted test suspension and buffered M/50 glucose. Optimum reduction of TTC generally occurred with concentrations of 0.05 and 0.1 per cent (Table I). Although 0.1 per cent TTC has been found to be toxic for growth and acid production of a number of bacterial species, including lactobacilli,[7] this concentration did not appreciably alter the metabolic rate of the washed cells of lactobacilli. Therefore, 0.1 per cent TTC was employed in future experiments.

TABLE I

THE RELATIONSHIP BETWEEN CONCENTRATION OF TETRAZOLIUM AND DEGREE OF REDUCTION BY LACTOBACILLI

LACTOBACILLUS STRAIN	CELL CONCENTRATION (MG. N/ML.)	FINAL TETRAZOLIUM CONCENTRATION (%)				
		0.01	0.05	0.1	0.15	0.2
		COLORIMETER READINGS				
L. casei	0.136	153	167	198	147	114
L3	0.490	135	152	187	130	80
L6	0.051	74	216	154	99	58
L7	0.053	318	239	246	120	16

Age of Culture.—If TTC is reduced by enzymatic activity within bacterial cells, it seems likely that the rate of its reduction might be influenced by the age of a culture. To test this hypothesis, lactobacilli strains were grown in 3 per cent phytone-2 per cent dextrose broth for varying periods between 6 and 48 hours and their ability to reduce TTC was tested at the end of each incubation period.

TABLE II

THE EFFECT OF CULTURE AGE ON TETRAZOLIUM REDUCTION BY LACTOBACILLI

LACTOBACILLUS STRAIN	CELL CONCENTRATION (MG. N/ML.)	AGE OF CULTURE IN HOURS				
		6	12	18	24	48
		COLORIMETER READINGS				
L. casei	0.160	156	20	31	50	6
L3	0.514	27	152	279	188	0
L6	0.050	77	266	244	129	0
L7	0.055	94	240	271	151	0

The data for 4 lactobacillus strains indicate that cells from 12- and 18-hour cultures reduce more TTC in 1 hour than do cells from 6-, 24-, or 48-hour cultures (Table II). It was difficult to remove formazan from the cells of 6-hour cultures, for some of it remained firmly bound even after acetone extraction. The actual amount of TTC reduced by 6-hour cells may have been equal to that reduced by older cells, but the true reducing activity of the younger

cells may not have been reflected in the colorimetric readings of their acetone extracts. On the basis of the above observations, however, 16- to 18-hour cultures were employed in succeeding experiments.

Hydrogen-ion Concentration.—Much of the early work on the reduction of tetrazolium by microorganisms utilized media buffered at approximately neutrality or on the alkaline side. However, detailed studies of specific microorganisms have revealed that the optimal pH for TTC reduction varies with experimental conditions. For instance, Kopper[8] found that maximal reduction of TTC by young cells of *Escherichia coli* occurred at approximately pH 7.5, while maximal reduction by old cells was observed in a more alkaline medium (pH 8.5). Since optimal reduction of TTC by bacterial cells varied with pH and the age of culture, it was necessary to establish optimal pH for its reduction by lactobacilli.

Optimal pH for reducing 0.1 per cent TTC was determined in a mixture of equal volumes of standard test suspension and buffered M/50 glucose. Tests were made at intervals of approximately one unit between pH 5 and 8. Under such conditions optimal TTC reduction varied with pH and the lactobacillus strains (Table III). However, when considering the entire group of lactobacilli, pH 6.0 was considered optimal for reduction of TTC.

TABLE III

THE INFLUENCE OF PH ON THE REDUCTION OF TETRAZOLIUM BY LACTOBACILLI

LACTOBACILLUS STRAIN	CELL CONCENTRATION (MG. N/ML.)	PH 5	PH 6	PH 7	PH 8
		COLORIMETER READINGS			
L. casei	0.136	490	215	143	94
L3	0.512	28	134	112	89
L6	0.050	233	354	168	10
L7	0.047	310	258	184	6

Effect of Temperature.—Since the metabolic activities of bacteria are influenced by temperature, its effect on the reduction of 0.1 per cent TTC by lactobacilli was tested at 37° C., 45° C., and 50° C. in an equal mixture of standard test suspension and M/50 glucose. Over-all optimal TTC reduction was obtained at 45° C., while it was less at 37° C. and 50° C. (Table IV).

Buffer Molarity.—The effect of electrolyte concentration on TTC reduction by lactobacilli was tested by the routine procedure, with buffer molarity ranging from 0.05 to 0.5. Increasing electrolyte concentration above 0.1 M had little influence on the rate of TTC reduction. The rate of TTC reduction was usually decreased in a buffer whose molarity was 0.09 or less. Subsequent test solutions were prepared with a 0.2 M phosphate buffer at pH 6.0.

Cell Concentration.—It is evident from the data presented in Tables I to IV, that the cell concentration producing a given amount of TTC reduction is not constant but varies widely with the strain of lactobacillus.

Before determining precisely the effect of cell concentration upon the rate of TTC reduction, it was necessary to determine endogenous reduction. A comparison was made of the reduction of TTC in the absence of glucose, by repre-

sentative homo- and heterofermentative lactobacilli (Table V). With the exception of *L. brevis*, the endogenous respiration was negligible for both groups of lactobacilli at the minimum cell concentration visibly reducing TTC in the presence of glucose.

TABLE IV

THE EFFECT OF TEMPERATURE ON TETRAZOLIUM REDUCTION BY LACTOBACILLI

LACTOBACILLUS STRAIN	CELL CONCENTRATION (MG. N/ML.)	TEMPERATURE		
		37° C.	45° C.	50° C.
		COLORIMETER READINGS		
L. casei	0.204	383	453	423
L3	0.450	427	542	462
L6	0.056	184	226	44
L7	0.053	185	330	93

TABLE V

THE MINIMUM CELL CONCENTRATION OF LACTOBACILLI REQUIRED TO PRODUCE DISCERNIBLE REDUCTION OF TETRAZOLIUM WITH AND WITHOUT GLUCOSE

LACTOBACILLUS STRAIN	FERMENTATIVE TYPE	MINIMUM CELL CONCENTRATION (MG. N/ML.) PRODUCING DETECTABLE REDUCTION	
		NO GLUCOSE	M/50 GLUCOSE
L. casei	homo-	0.88	0.08
L3	homo-	0.72	0.28
L6	hetero-	0.76	0.008
L7	hetero-	0.44	0.009

TABLE VI

THE EFFECT OF CELL CONCENTRATION ON THE REDUCTION OF TETRAZOLIUM BY LACTOBACILLI

LACTOBACILLUS STRAIN	FERMENTATIVE TYPE	MINIMUM CELL CONCENTRATION (MG. N/ML.) PRODUCING DETECTABLE REDUCTION
L. casei	homo-	0.08
L. acidophilus	homo-	0.80
L. delbrueckii	homo-	0.20
L3	homo-	0.28
L5	homo-	0.08
L. brevis	hetero-	0.04
L. fermenti	hetero-	0.009
L4	hetero-	0.009
L6	hetero-	0.008
L7	hetero-	0.009
L8	hetero-	0.01
L9	hetero-	0.01
L10	hetero-	0.01
L11	hetero-	0.01
L12	hetero-	0.009

To determine more precisely the concentration of cells of each species required to produce a detectable reduction of TTC, dilutions of the standard test suspension, ranging in nitrogen concentration from 0.007 to 0.8 mg. N/ml., were tested for reducing activity by the standard procedure utilizing M/50 glucose. Ten of the 15 lactobacilli strains reduced TTC in cell concentrations near 0.01 mg. N/ml., whereas the remaining 5 strains were considerably less active, requiring from 0.08 to 0.80 mg. N/ml. for reduction (Table VI). Based on the minimum cellular concentration required to reduce tetrazolium, the strains and

species fell into two groups. The strains requiring a higher or lower cell concentration for discernible reduction of TTC were those previously classified as homo- and heterofermentative, respectively. Thus, it appears that the minimum cell concentration which will produce detectable reduction of TTC is significantly different between homo- and heterofermentative lactobacilli.

DISCUSSION

Studies of the factors which influence reduction of TTC by washed cells of homo- and heterofermentative lactobacilli indicate that TTC concentration, age of culture, hydrogen-ion concentration, temperature, and cell concentration all affect the rate of reduction of TTC. Buffer concentration, in the range of 0.1 to 0.5 molarity, had little influence on TTC reduction. Thus, it was found that optimal reduction of TTC by most strains of both metabolic types of lactobacilli occurred with an 18-hour culture in 0.2 M buffer at pH 6.0, with 0.1 per cent TTC and at 45° C. While the rate of reduction of TTC varied with the lactobacillus strain, optimal conditions for TTC reduction were essentially the same for both homo- and heterofermentative lactobacilli. The similarity of optimal conditions indicates that the same factors in each metabolic type are probably concerned in the reduction of TTC but that the amount of these factors varies consistently with the metabolic types. Minimum cell concentration, as determined by nitrogen content, which will discernibly reduce TTC is consistently less for the heterofermentative strains than it is for the homofermentative strains. The rate of TTC reduction increases very rapidly as the cell concentration of heterofermentative lactobacilli in the test solution is increased. The rate of TTC reduction increases slowly as the cell concentration of the homofermentative lactobacilli is increased above the minimum amount that will cause detectable reduction. Thus, while the optimal rate of TTC reduction by each of the metabolic types of lactobacilli is affected in a like manner by the same factors, the amount of the substance causing TTC reduction appears to be consistently different in the two types.

The reduction of TTC by microorganisms has usually been attributed to enzymatic activity.[8-11] The apparent redox potential of TTC is about –0.08 V, which has led to the conclusion that its reduction is probably caused by dehydrogenase systems requiring coenzymes I or II.[12] While DPN or TPN linked dehydrogenases, as well as other dehydrogenases, may be associated indirectly with the reduction of TTC, it is more likely that flavin enzymes are the direct mediators of its reduction.[13, 14] What enzymatic systems are associated with the reduction of TTC by lactobacilli has not been determined. Our attempts to demonstrate a flavin enzyme system in lactobacilli have been negative.

Several of the conditions of optimal reduction of TTC by intact lactobacilli cells are different from those affecting isolated enzyme systems from yeast or from *E. coli*, which might indicate that different systems are functioning in different microorganisms. For instance the isolated flavin enzymes are most active in an alkaline medium (pH 8.3 for yeast diaphorase, and pH 7.8 for flavoprotein from *E. coli*),[12, 13] but the optimal pH for the reducing activity of the lactobacilli was 6.0 or even as low as 5.0. These findings do not necessarily indi-

cate that different enzymes are functioning, for in one instance isolated enzymes are involved and in the other instance the activity of intact bacterial cells are being measured.

The reduction of TTC is a possible method for differentiating homo- from heterofermentative lactobacilli since there is a clear-cut differentiation between the two groups based upon the minimum cell concentration required to cause discernible reduction of TTC in the presence of glucose. In a typical test, heavy cell suspensions of both homo- and heterofermentative lactobacilli reduce TTC to red formazan by the method described, indicating the potential reducing ability of each metabolic type. With the proper dilution of cells, however, heterofermentative lactobacilli markedly reduces TTC, while homofermentative lactobacilli showing little or no reducing activity.

SUMMARY

A study has been made of the reduction of 2, 3, 5-triphenyltetrazolium (TTC) by washed cells of 15 strains and species of the genus *Lactobacillus*, representing both homo- and heterofermentative types. Maximal reduction occurred with 0.1 per cent TTC added to equal volumes of an 18-hour culture and M/50 glucose in 0.2 M buffer (pH 6.0) and incubated at 45° C. The minimum cell concentration, based on nitrogen content, which would effectively reduce TTC was less for heterofermentative strains than it was for homofermentative strains. The reduction of TTC is suggested as a means of differentiating homo- and heterofermentative strains of lactobacilli.

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THE REDUCING PROPERTY OF SALIVA TOWARD SEVERAL OXIDATION-REDUCTION INDICATOR DYES

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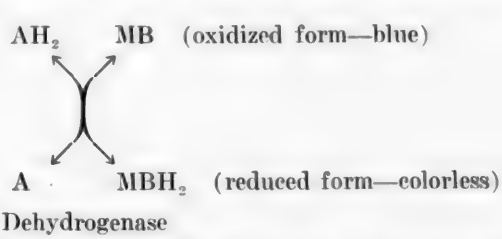
REDUCIBLE dyes such as methylene blue have been used extensively to study the catalytic transfer of hydrogen (dehydrogenation) by animal and plant tissues and by bacteria.[14] If oxidized methylene blue is added to a suspension of chopped muscle, brain, or kidney tissue, or a suspension of bacteria in the presence of a suitable substrate, the dye is rapidly reduced to a colorless state.[11] The reduction is an indication of the presence of dehydrogenases. The reaction with methylene blue (MB) may be expressed as follows:



There are present in whole saliva biologic systems capable of reducing certain oxidation-reduction indicator dyes at varying rates. Stuteville[13] first observed that saliva will reduce some dyes, such as indophenol, to a colorless state. He attributed this reducing power to the ascorbic acid in saliva. Zimmet and Dubois[15] recorded anew that saliva will reduce indophenol. Pincus[9] by using petroleum jelly over saliva and Thunberg tubes investigated the reducing property of saliva toward several redox indicators, finally settling on indophenol (o-chlorophenol indo-2:6 dichlorophenol). He found that ascorbic acid will reduce the reduction time of saliva toward indophenol by 50 per cent. Reduction time was shortened by the addition of a gram-positive bacillus. Potassium cyanide and methane, at 0.001 M concentrations, increased the time required to reduce indophenol by saliva from 5.5 to 20 minutes. Pincus noted that animal saliva (monkey, dog, and rabbit) did not reduce indophenol. Eisenbrandt[4, 5] measured the Eh of saliva by using an Eh potentiometer with platinum electrode and found that, on standing, the potential in saliva drifted to the negative scale. The initial oxidation-reduction potential of unstimulated saliva of 5 subjects had a mean value of + 261 mv.

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The purpose of this paper is to report on the general properties of this phenomenon by using several redox dyes and to determine what fraction of the saliva contains the active components.

METHODS AND MATERIALS

Preliminary experiments were carried out employing standard Thunberg tubes and following the methods established by previous investigators.[14]

It was found that these technics were not suitable for quantitative measurements in this laboratory. It was difficult to maintain anaerobic conditions with many of the available Thunberg tubes. More important was the fact that sedimentation of the saliva greatly influenced the rate of reduction of the dye. The Thunberg technic was therefore abandoned in favor of the simplified method of Friedeman and Hollander.[6]

In this method, instead of using evacuated tubes, the reagents were mixed with melted agar and the reaction observed in the solidified medium. Thus anaerobic conditions were obtained and sedimentation of the solids was prevented. A large number of simultaneous determinations were possible by this technic. The reagents used were as follows: (1) 2 per cent agar containing 0.5 per cent dibasic sodium phosphate and adjusted to pH 7.1; (2) 0.0005 M methylene blue, or other indicator dye; (3) paraffin-stimulated saliva, collected between 10 and 11 A.M.

The procedure consisted of melting the agar and pipetting 3.0 ml. into test tubes (100 mm. by 10 mm.). When the agar had cooled to 42° C., 0.5 ml. of methylene blue or other dye, and 0.5 ml. of saliva were added (all previously warmed to 37° C.). Complete admixture of the components was accomplished by immediate shaking. The tubes were plunged at once into a freezing mixture of ice and salt for 1 minute, then placed in a water bath at 37° C. and the change in color of the dye was observed. Reduction of methylene blue is not a linear function,[10] hence all tubes were read at a point of 90 per cent decoloration. The slight diffusion of oxygen from the air into the agar caused a well-demarcated zone of blue coloration near the top.

DEFINITION OF UNIT ACTIVITY

The reducing coefficient (R. C.) of saliva with respect to redox dyes was defined as the reciprocal of the time in minutes required by 0.5 ml. of saliva to reduce to a point of 90 per cent decolorization, a 0.5 ml. of 5×10^{-5} M. solution of methylene blue (or other indicator dye where stated) under standard conditions of temperature and pH.

EXPERIMENTAL FINDINGS

1. *Reducing Property of Saliva Toward Several Redox Indicators.*—The reducing property of saliva toward the oxidation-reduction indicators whose E° values (potentials of half-reduced dyes at pH 7.0) cover the range of most biologic enzyme systems was tested. The indicators selected were toluylene blue ($E^{\circ} = -0.115$v), methylene blue ($E^{\circ} = 0.011$v), 2, 3, 5 triphenyl tetrazolium chloride ($E^{\circ} = -0.08$v), cresyl violet ($E^{\circ} = -0.170$v) and safranine

($E^{\circ} = -0.289v$). The relative position of the various dyes with respect to E° at a pH 7.0 at 30° C. is illustrated in Fig. 1. The behavior of one system toward another may be determined from the scale. Thus the reduced form of any indicator will reduce the oxidized form of all indicators more positive to it, while the oxidized form will oxidize the reduced forms of more negative systems.

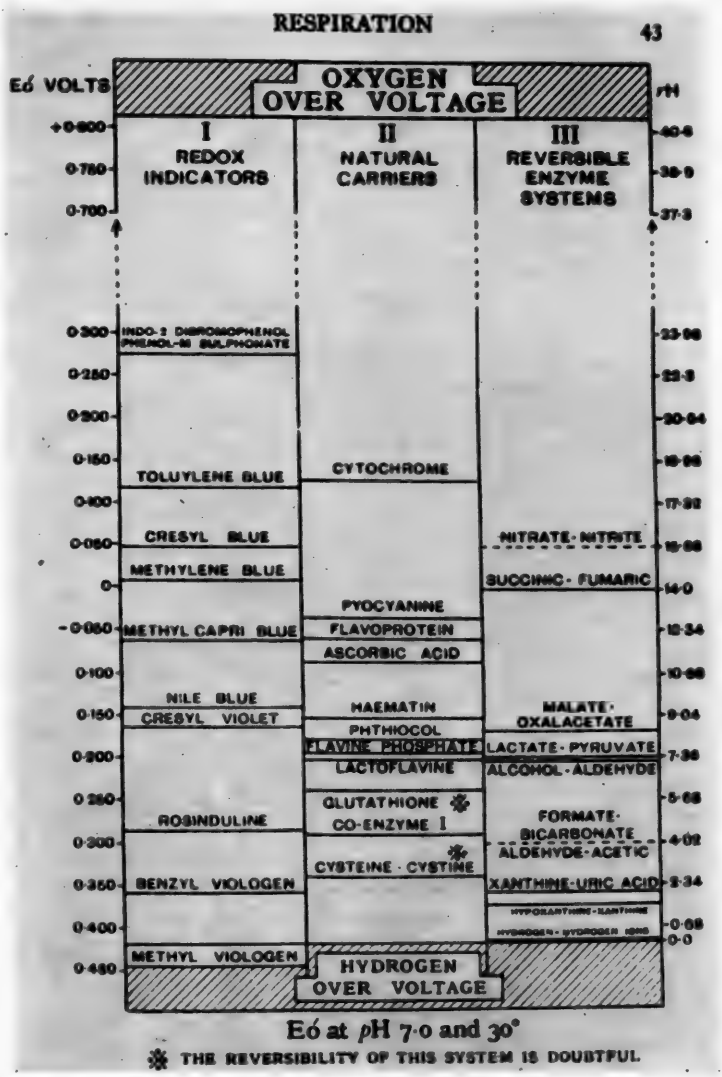

Fig. 1.—The E° at pH 7.0, 30° C. of some redox indicators and reversible enzyme systems. (From Stephenson, Bacterial Metabolism, Longmans, Green & Co.)

Table I indicates the reducing coefficient of saliva from laboratory personnel toward several dye indicators.

TABLE I
THE REDUCING COEFFICIENT × 100 OF THE SALIVA OF 12 LABORATORY PERSONNEL IN THE PRESENCE OF SEVERAL DIFFERENT DYE INDICATORS (5×10^{-4} M)

DYE INDICATOR	E°	REDUCING COEFFICIENT × 100											MEAN
Toluylene blue	+.115	8.3	4.3	3.1	14.3	8.3	10.0	3.7	6.2	2.2	3.1	5.2	2.4	5.92*
Methylene blue	+.011	7.2	2.8	1.8	9.8	5.2	7.1	2.2	4.3	1.3	1.7	4.1	1.7	4.10*
2, 3, 5 triphenyl tetrazolium chloride	−.08	Light pink color produced in all samples between 15 and 60 minutes												
Cresyl violet	−.017	6.6	2.0	1.0	6.2	3.2	5.5	1.8	3.8	0.8	1.2	3.0	0.9	3.00*
Safranin	−.289	Slightly reduced after incubation overnight (10 to 15 hours)												

*The mean differences in the reducing coefficients are significant ($P < .01$).

2. *Effect of Boiling Saliva on Reducing Coefficient.*—Boiling a saliva specimen for 5 minutes destroys its ability to reduce the above-mentioned redox indicators.

3. *Separation of Active Fraction From Whole Saliva.*—When saliva is permitted to stand at room temperature for 30 minutes, the activity (approximately 60 per cent) is largely recovered in the sediment.

Saliva passed through a Seitz filter loses its ability to reduce redox indicators.

A sample of pooled saliva was centrifuged for 15 minutes at a speed of 3,300 r.p.m. The sediment was resuspended in an equal volume of 0.1 M phosphate buffer pH 7.1. The activity of the supernatant and the sediment was examined—Approximately 70 per cent of the activity was found in the sediment.

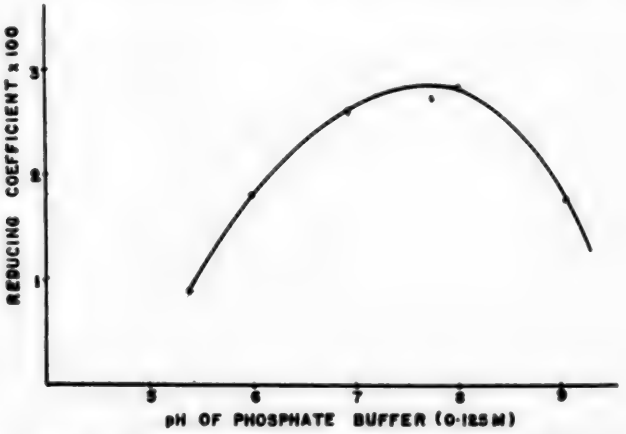

Fig. 2.—Effect of pH on the reducing coefficient of a pooled sample of saliva toward methylene blue (5×10^{-4} M).

4. *Effect of pH on the Reducing Coefficient of Pooled Saliva.*—A 2 per cent agar medium was made with 0.125 M solution of phosphate buffer. According to the procedure previously described, 0.25 ml. methylene blue (5×10^{-4} M) and a 0.20 ml. aliquot of saliva from a pooled specimen was added to the buffered agar media. The effect of pH, using a phosphate buffer, on the reducing coefficient of a pooled sample of saliva is shown in Fig. 2. Maximum activity was found at pH 7.5 to 8.0.

5. *Correlation Between the Amount of Sediment in the Saliva and Its Reducing Coefficient.*—The amount of sediment (epithelial cells, bacteria, leukocytes, and unidentified debris) in a sample of saliva was determined by the use of "Westergren" sedimentation tubes. A measured quantity of saliva (1 ml.), drawn into the tube, was placed in a suitable stand, and the amount of sediment read in the sedimentation tube units at one-hour intervals. During the sedimentation of saliva the height of the column decreases rapidly the first two hours or more due to the packing of the sediment. Further settling after this period occurs very slowly. Final readings are taken when the height of the column is approximately constant.

There is a significant positive linear correlation ($r = 0.811$) between the total sediment in saliva samples and the corresponding reducing coefficients as illustrated in Fig. 3.

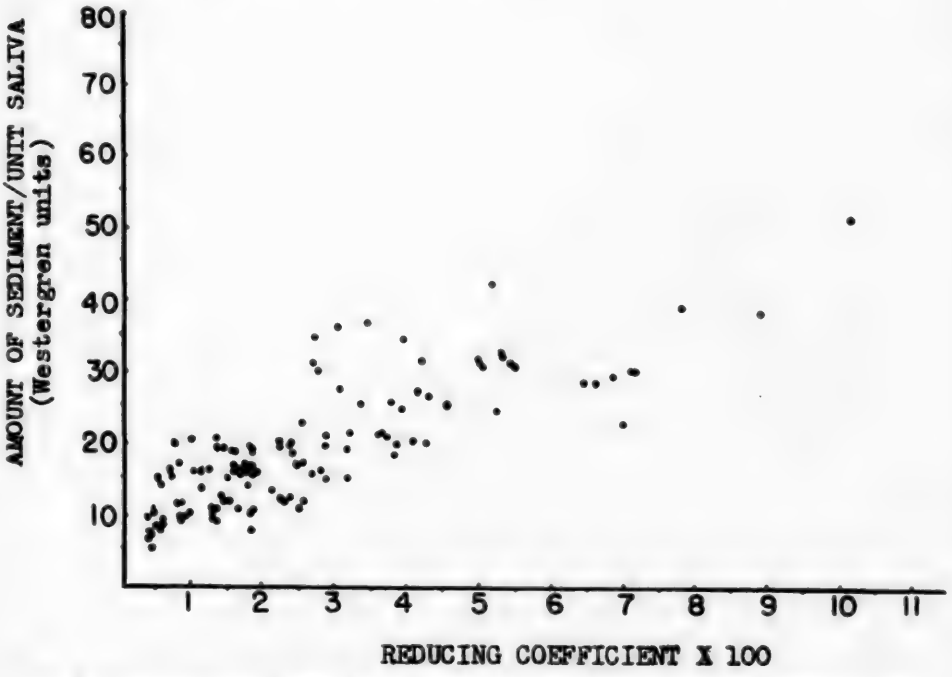

Fig. 3.—The relationship between the amount of sediment and the reducing coefficient ×100, per unit of saliva, in 114 children, aged 7 to 10 years.

6. *Variation in Reducing Coefficient of Saliva Obtained From Different Individuals.*—Paraffin-stimulated saliva was collected from 114 children whose ages ranged from 7 to 10 years. The reducing coefficient ranged from 0.4 to 10.7 units. The mean value was 2.6 units. The observed distribution of reducing coefficients cannot be fitted to a normal probability distribution curve ($x^2 = 25.68$; $P < .001$). (Fig. 4.)

7. *The Reducing Coefficient of Caries-Free Children.*—A total of 29 caries-free children ranging in age from 12 to 15 years were selected from two cities,

with another 29 randomly selected children serving as controls. The mean reducing coefficient of the saliva from the caries-free group was 3.01 with a standard error of ± 0.59 as compared to a mean value of 2.85 ± 0.48 for the control groups. The differences are not significant.

8. *Effect of Inhibitors on Reducing Coefficient of Saliva.*—Furacin (5-nitrofuraldehyde semicarbazone) has been shown to inhibit many dehydrogenases, particularly those involved in carbohydrate metabolism.[2] Furacin, after incubation with saliva for 1 hour, inhibited reduction of methylene blue (5×10^{-4} M) at pH 7.1 by 50 per cent at a concentration of 4.2×10^{-5} M. A 50 per cent lowering in reducing coefficient was obtained by a 7.3×10^{-6} M solution of sodium cyanide, and a 1.3×10^{-4} M solution of iodoacetate at pH 7.1. No inhibitory effect was observed by 0.001 M. concentrations of sodium fluoride, urethane, or penicillin (5,000 units).

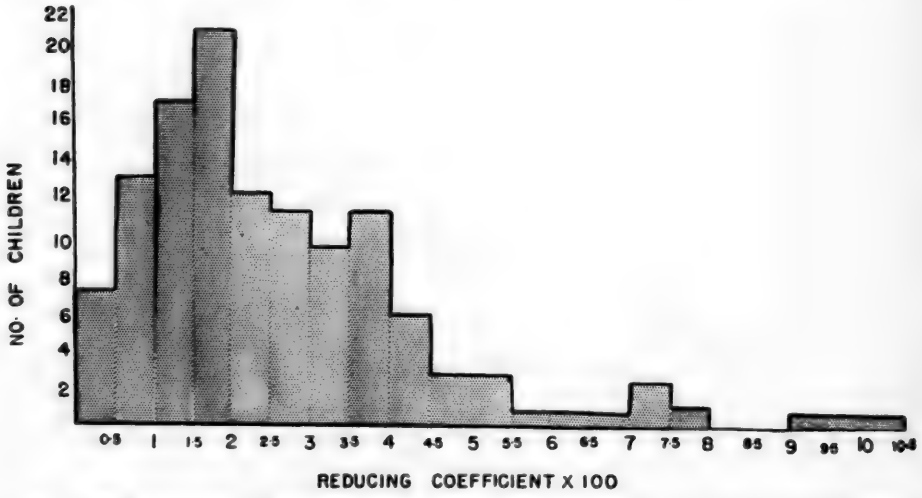

Fig. 4.—The distribution of the reducing coefficient, × 100, of saliva samples from 114 children, aged 7 to 10 years.

9. *Effect of Anions on the Reducing Coefficient of Saliva.*—No demonstrable effect on the reducing coefficient of saliva was observed by addition of lactate, glutamate, succinate, citrate, or formate in concentrations of 1×10^{-3} M, pH 7.1. The reducing activity was measured one-half hour after addition of the substrate to the saliva. Similarly, no demonstrable effect was noted when the above substrates were added to the centrifuged sediment washed twice with isotonic saline solution.

DISCUSSION

As a preface to the discussion of experimental findings something should be said of the limitations of the use of oxidation-reduction potential indicators. Saliva is the very antithesis of a pure chemical compound and it would indeed be difficult to prepare or imagine a more heterogeneous mixture. The investi-

gator, as stated by W. M. Clark,[3] "makes painfully exact measurements upon frightfully impure compounds." In measuring oxidation-reduction potentials of biologic systems, there are also difficulties: (1) in obtaining reproducible results, since no 2 aliquots of saliva, even from the same sample, are identical. (2) in knowing what constituents of the system are instrumental in the changes observed, and (3) in determining the effects of certain components of the system which indirectly by catalytic, poisoning, or other activities exert an influence on the potentials.[7]

However, measurement of oxidation-reduction potential of saliva is an attempt at a study of a dynamic rather than a static state; of a composite whole rather than an isolated factor. Some interesting facts emerge from a study of the redox systems in saliva.

Table I indicates that saliva readily reduces the oxidized form of oxidation-reduction indicators whose E° at pH 7.0 is more positive than approximately –0.20v. The relative speed at which saliva reduces equimolar solutions of the redox indicators is indicated by the following series: toluylene blue is reduced faster than (>) methylene blue > 2, 3, 5 triphenyl tetrazolium chloride > cresyl violet > safranin. It is interesting to note that the E° (eg., –0.20v) below which the redox indicators are not readily reduced by saliva corresponds to the lactate-pyruvate system (Fig. 1). This system at pH 7.0 will reduce all indicators more positive than –0.20v but none that are more negative.

The inability to demonstrate an increase of the reducing coefficient of saliva upon addition of substrates as lactate, glutamate, succinate, citrate, or formate, is probably explained on the basis that a sufficient amount of oxidizable substrate is present, even in the washed sediment, which when metabolized reduces all the methylene blue. Thus, 1 ml. of 5×10^{-4} M methylene blue is approximately equivalent to 0.09 mg. of glucose, assuming 1 ml. glucose donates 2 atoms of hydrogen. Such minute amounts of glucose or other hydrogen donators are probably found in whole saliva or the washed sediment of saliva.

The loss of activity of saliva that is passed through a Seitz filter, or from the supernatant of saliva that is centrifuged, and the recovery of approximately 70 per cent of the reducing activity in the sediment strongly indicates that the reducing fraction is primarily associated with the solids present in saliva. The correlation between the amount of sediment and the corresponding reducing coefficient ($r = 0.811$) made it possible visually to divide saliva into high and low reducing coefficient groups on the basis of the amount of sediment.

Further identification of the component of saliva associated with its reducing activity must await separation of the sediment into its components. The sediment in saliva consists of bacteria, desquamated cells, leukocytes, and some debris. It is probable that the reducing coefficient of saliva is correlated with one or more of the above components, most probably the total bacterial populations. General oral hygiene conditions determine to a large extent the total number of bacteria in the mouth[1]; hence the possibility that the reducing coefficient, as defined, may be correlated with the state of oral hygiene.

CONCLUSIONS AND SUMMARY

1. Saliva reduces redox indicators whose E^0 at pH 7.0 is more positive than –0.20v. The rate at which equimolar solutions of the indicators are reduced is indicated by the following series: toluylene blue faster than (>) methylene blue > 2, 3, 5 triphenyl tetrazolium chloride > cresyl violet > safranin (only slightly reduced).

2. The reducing coefficient (× 100) of saliva in 114 children 7 to 10 years of age ranges from 0.4 to 10.7 units. The observed reducing coefficients do not fit a normal probability distribution curve.

3. The reducing coefficient is decreased 50 per cent at pH 7.0 by 4.2×10^{-5} M Furacin; 7.3×10^{-6} M cyanide and 1.3×10^{-4} M iodoacetate. Sodium fluoride, penicillin, and urethane had no inhibitory effect.

4. No demonstrable effect by adding the following oxidizable substrates was observed: citrate, lactate, glutamate, succinate, and formate.

5. The reducing coefficient of saliva is not sharply influenced by pH. A broad pH optimum exists between 7 and 8.

6. The reducing activity of saliva is associated with the insoluble sediment. This fraction is composed of bacteria, desquamated epithelial cells, leukocytes, and debris. It appears that the reducing coefficient of saliva may be primarily a function of one of the above components.

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TITRATABLE ACIDITY AND TITRATABLE ALKALINITY OF THE SALIVA OF CASES CHOSEN WITH REFERENCE TO DENTAL CARIES

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SIX groups of workers[1-6] have, in the past fifty years, observed an association between titratable alkalinity of the saliva (or the buffer capacity of the saliva to acid addition) and the incidence of dental caries. The 315 individuals reported on by Turner, Scribner and Bell[6] were a random sampling of the Greater Boston school population examined at the Forsyth Dental Infirmary.

An extension of these studies is described in this paper. Emphasis was placed on children with extensive, active, rapid dental caries and as large a sample of children whose teeth were free from dental decay as was available. Mirror, probe, and radiographs were used in the dental examinations.

METHODS AND SUBJECTS

For 213 of the children, the method used by Turner, Scribner, and Bell was employed. The added acid for determining alkalinity was 0.001 N HCl; the indicator was methyl red. The added alkali for determining acidity was 0.001 N NaOH; the indicator was phenolphthalein.

For a third group (200 ± 2 children), the method used was with the same added solutions; but the end points were determined on the Beckman pH meter set at 5.5 and 5.0 for titratable alkalinity, and at 8.5 and 9.0 for titratable acidity.

RESULTS

Fig. 1 shows the frequency distribution for titratable acidity for the 213 children, using a color indicator for end points; Fig. 2 shows such a distribution for the 202 children using pH 8.5 on the Beckman pH meter as the end point.

Figs. 3 and 4 show, respectively, frequency distributions for titratable alkalinity for the 213 children using a color indicator for the end point and for the 198 children using pH 5.5 on the Beckman pH meter as the end point.

It is of interest that the acidity values in these 2 series are quite similar in range and distribution to each other and not unlike the random sampling distribution of the 315 previously reported cases.[6]

The dental examinations were with mirror and probe by one examiner for each series and were supported by bite-wing radiographs.

In characterizing the condition of the teeth for the 213 cases, 3 categories were used: caries-free, moderate decay (1-20 DMF surfaces), extensive decay

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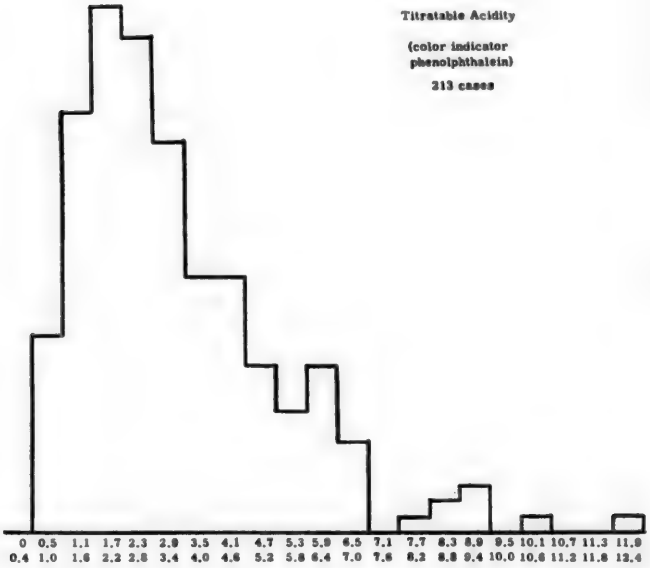

Fig. 1.—Titratable acidity (color indicator, phenolphthalein); 213 cases.

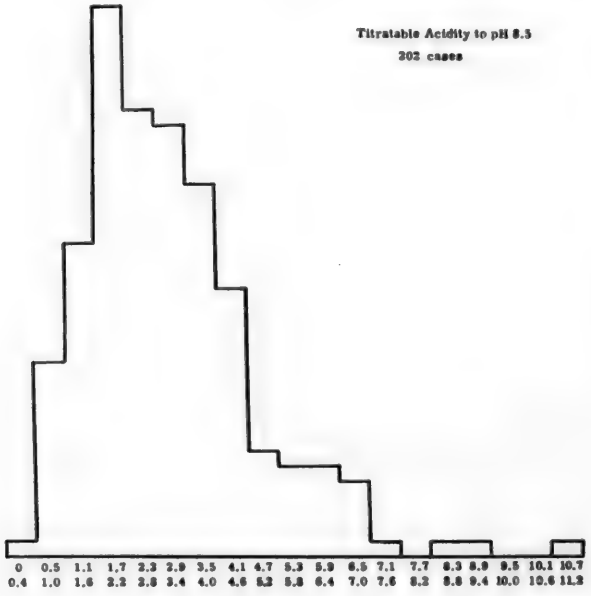

Fig. 2.—Titratable acidity to pH 8.5; 202 cases.

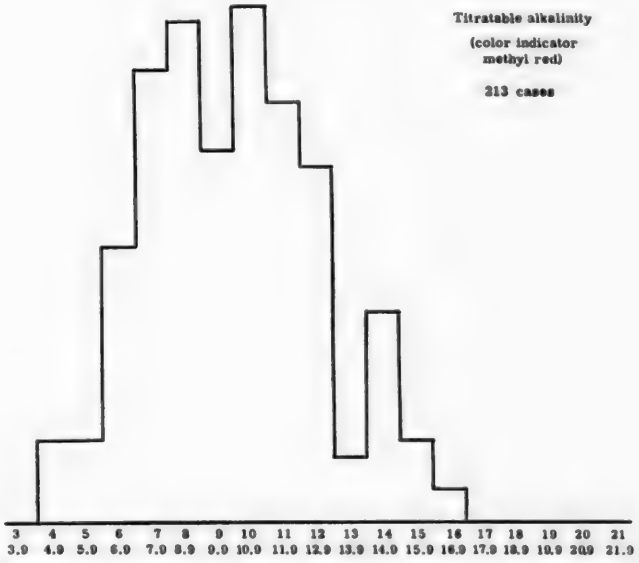

Fig. 3.—Titratable alkalinity (color indicator, methyl red); 213 cases.

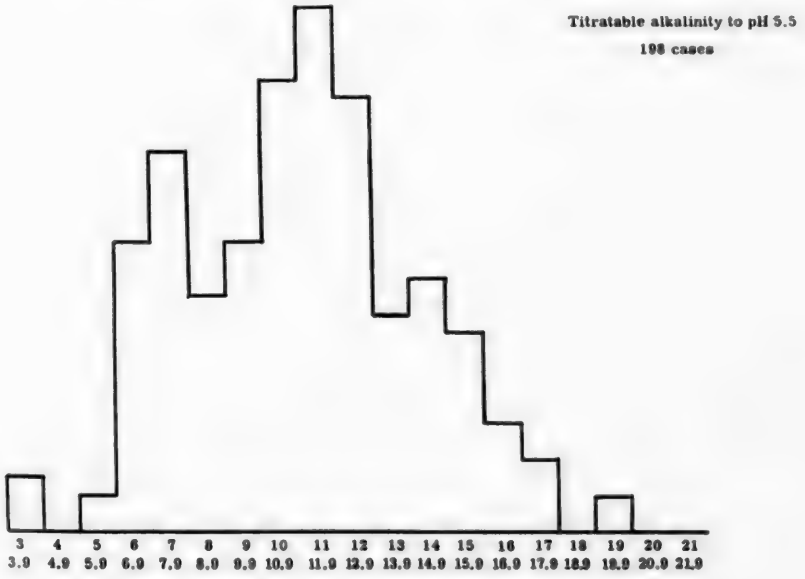

Fig. 4.—Titratable alkalinity to pH 5.5; 198 cases.

(21 and more DMF surfaces). The statistical findings for each category with respect to titratable acidity are shown in Table I, and those for each category with respect to titratable alkalinity in Table II.

TABLE I
TITRATABLE ACIDITY—PHENOLPHTHALEIN INDICATOR

	NUMBER	MEAN	SIGMA
Caries-free	29	2.28	1.39
Moderate	103	3.15	1.76
Extensive	81	3.95	2.33

TABLE II
TITRATABLE ALKALINITY—METHYL RED INDICATOR

	NUMBER	MEAN	SIGMA
Caries-free	29	10.87*	2.30
Moderate	103	10.66	2.48
Extensive	81	8.32	2.15

*This figure is not quite in line with those obtained for caries-free cases in the other 2 series, namely, 13.14 and 12.34.

For the 200 ± 2 cases, 7 categories were used in an attempt to assess not only past caries experience in terms of total DMF surfaces, but also to take into account current or recent dental decay.[7] The findings are summarized in Tables III-V.

TABLE III
TITRATABLE ACIDITY TO pH 8.5 AND pH 9.0

CLASSIFICATION OF DENTAL DECAY	NUMBER	TO pH 8.5		TO pH 9.0	
		MEAN	SIGMA	MEAN	SIGMA
Caries-free	29	2.57	1.47	6.40	3.28
Moderate Static	10	3.43	1.33	7.72	2.63
Moderate Active Slow	62	2.73	1.43	6.48	2.59
Moderate Active Rapid	33	3.15	1.49	6.68	2.56
Extensive Static	2	2.10	—	6.30	—
Extensive Active Slow	7	3.76	2.12	7.93	3.13
Extensive Active Rapid	59	3.60	1.94	7.50	3.05

TABLE IV
TITRATABLE ALKALINITY TO pH 5.5

CLASSIFICATION OF DENTAL DECAY	NO.	MEAN	SIGMA
Caries-free	28	12.34	2.78
Moderate Static	10	13.36	2.72
Moderate Active Slow	60	11.72	2.51
Moderate Active Rapid	33	10.93	2.85
Extensive Static	2	11.00	—
Extensive Active Slow	7	10.73	2.59
Extensive Active Rapid	58	9.47	3.02

TABLE V
TITRATABLE ALKALINITY TO pH 5.0

CLASSIFICATION OF DENTAL DECAY	NO.	MEAN	SIGMA
Caries-free	29	14.87	2.92
Moderate Static	10	15.77	3.38
Moderate Active Slow	62	13.84	2.78
Moderate Active Rapid	34	12.68	3.36
Extensive Static	1	14.40	—
Extensive Active Slow	7	13.13	2.89
Extensive Active Rapid	59	11.76	3.34

Inspection of Tables I and III, and II and IV, reveals that, with color indicator or pH meter to comparable end points, the caries-free cases, as a group, show lower acidity and higher alkalinity than those cases with extensive decay, which show higher acidity and lower alkalinity.

In order to find out whether any statistically significant relationship exists between the groups chosen on the basis of dental caries experience (within each series), "t" tests were applied. Tables VI and VII show "t" values for acidity and alkalinity for the series employing the pH meter.

Under the conditions of these tests and using past experience as the index of dental caries, it would appear that even more closely associated with dental decay than acids themselves, as found in "resting" saliva, is the capacity of the "resting" saliva to buffer against acids.

TABLE VI
TITRATABLE ACIDITY

	"t" TO 8.5	PROB.	"t" TO 9.0	PROB.
Caries-free vs. Moderate	1.16	0.10	0.43	n. s.
Caries-free vs. Extensive	2.45	0.02	1.58	0.10
Moderate vs. Extensive	2.45	0.02	1.99	0.05
Rapids vs. Slows	2.21	0.05	1.22	n. s.

TABLE VII
TITRATABLE ALKALINITY

	"t" TO 5.5	PROB.	"t" TO 5.0	PROB.
Caries-free vs. Moderate	1.23*	n. s.	1.86*	0.10
Caries-free vs. Extensive	4.06	0.01	4.07	0.01
Moderate vs. Extensive	4.42	0.01	3.37	0.01
Rapids vs. Slows	3.51	0.01	3.33	0.01

*Caries-free vs. Moderate Active Rapid (eliminating Moderate Static and Moderate Slow) "ts" = 1.92 and 2.70.

DISCUSSION

The above results suggest that it may not be so much the presence of acid as the absence of physiologic buffers on the alkaline side to handle these acids (and those from CHO degradation) that is associated with dental decay.

Dreizen, Reed, Niedermeier, and Spies[8] have reported differences in ion concentrations in salivas that have been obtained with and without paraffin stimulation. It should be emphasized that the saliva, used in the studies here reported, was in all cases resting saliva, i.e., nothing was taken into the mouth to stimulate salivary flow.

SUMMARY AND CONCLUSION

Titratable acidity and titratable alkalinity of the saliva are reported for a series of 213 children. This series does not represent a random sampling but rather emphasis was placed on selection of children who were either caries free or had experienced extensive dental decay. A large group of intermediate cases was also included. End points for titrations for this series were by color indicator.

Titratable acidity and titratable alkalinity of the saliva are also reported for a series of 200 ± 2 cases. Emphasis was placed on selection of children who were caries free or who had experienced extensive, active, rapid dental decay. Here titrations were to fixed pH values on the Beckman pH meter.

Frequency distributions for these 2 series are quite similar to each other. In the case of acidity they are quite similar to a random sampling series previously reported. In the case of alkalinity these 2 series, chosen with reference to extremes in dental decay experience, show greater tendency to multiple peaks than does the frequency distribution for the random sample.[6]

Judged on the basis of "t" values, titratable alkalinity appears to be more consistently related to dental decay than does titratable acidity.

ADDENDA

Since submitting this study for publication, a fourth series of children (222) has been studied for titratable acidity and titratable alkalinity of the saliva. The results of this series confirm the earlier 3. In addition, for this series titratable acidity was carried to pH's 8.5, 9.0, and 9.5, and titratable alkalinity was carried to pH's 6.5, 6.0, 5.5, 5.0, 4.5, 4.0, and 3.5, without finding an interval showing closer association with dental caries experience than those previously used; namely, pH 8.5 for titratable acidity and pH 5.5 for titratable alkalinity.

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A SIMPLE, RAPID METHOD FOR QUANTITATIVE DETERMINATION OF SALIVARY AMYLASE

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SALIVARY amylase offers considerable promise as a laboratory test for the estimation of caries activity, primarily because of reaction speed, but its exact role in the caries process remains controversial. The divergent claims which have arisen about this relationship stem, in our opinion, from the different analytical methods employed. Thus, studies[1-3] based on qualitative procedures (spot-plate methods) have given a high degree of correlation with DMF, whereas quantitative technics[4, 5] have not. The inaccuracies of the spot-plate method resulting from a visual observation of color change (dextrinizing time) and the uncontrolled environment made this method unsatisfactory for our purpose. On the other hand, the cumbersomeness of measuring reducing substances formed by the action of alpha-amylase on starch eliminated this technic for volume work. Our basic need was a simple, rapid quantitative procedure which would allow reasonably accurate analyses of large numbers of specimens of saliva. We wish to report a method which proved satisfactory for this purpose.

The standard method of reference for estimation of serum amylase is that of Somogyi[6] in which the amount of reducing substances formed by enzymatic action on a known amount of starch is determined quantitatively (copper reduction) and the results expressed as units of α-amylase. Most subsequent procedures, e.g., Van Loon's,[7] have measured the starch-iodine color changes which reflect the amount of residual starch remaining after amylase attack in terms of a standardized calibration curve. Carroll and Van Dyk[8] recently reported a method which employed congo red as an absorption indicator. None of these technics was satisfactory for rapid analysis of multiple samples because so much glassware or time was required.

Attempts to adapt these volumetric methods for our purpose also failed because the amount of starch necessary (240 to 250 mg.) for the quantity of enzyme present in 0.1 ml. of saliva did not give color intensities following the Beer-Lambert absorption law. This outcome was expected in light of the work of Boutarie and Anglade-Thevenet[9] who showed lineation for the starch-iodine color only in dilute concentrations of these reagents and then only in the

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region of maximum absorption (550 mμ). A systematic study of the variables in the amylase-starch reaction, combined with an accidental observation giving reproducible photometric values led to the development of a quantitative method satisfying the following specifications: (1) an easily measured volume of saliva, (2) incubation of saliva-starch mixture in a single test tube, (3) short incubation time, (4) arresting of enzymatic action and development of color in the same tube, (5) one transfer for photocolorimetric reading, and (6) comparison against the action of an amylase standard.

It was thought of interest to report our study of the factors influencing this reaction with special reference to the unplanned but fortuitous incident which actually provided the solution to our problem.

a. *Volume of saliva:* The smallest volume of saliva which we could readily measure for routine quantitative work was found to be 0.1 ml.

b. *Starch:* Mallinckrodt's analytical reagent grade starch was used which permitted the comparison of the different analyses since we found very little variations between lot numbers of this chemical. Empirically it was found that 19 ml. of 1.28 per cent solution of this reagent provided sufficient substrate for determination of the maximum amount of alpha amylase in 95 per cent of the specimens tested. Furthermore, when this suspension was buretted into test tubes and stoppered, it remained stable at room temperature (20° C.) at least 5 days in contrast to the rapid deterioration of starch left in large volumes. The advantage of this tube technic should have practical value in other laboratory procedures. Even though we found this starch contained between 9 to 10 per cent water, it was not believed necessary to dry it since the blank, standard, and unknown were all determined from the same preparation.

c. *Incubation time:* With the volumes of saliva and starch used it was possible to select an incubation time of 5 minutes, which is an agreement with Ericsson[10] whose data showed maximum enzymatic hydrolysis of starch occurring in the first 5 minutes of the reaction. This abbreviated time interval was a major factor in permitting 2 people to perform 150 determinations in a working day.

d. *Arresting of enzymatic activity and development of color:* This was accomplished by the addition of a reagent which contained HCl acid and the dilute working solution of iodine. The addition of acid was necessary because the low concentration of I_2 used for color development would not stop the amylolytic attack.

e. *Amylase standard:* We were successful in establishing a standard reading directly in milligrams of alpha-amylase. This resulted from the accidental discovery that a linear curve was obtained when 243 mg. of starch were incubated with commercial amylase; this curve closely paralleled the one obtained by serial dilution of an initial mixture of starch (243 mg.) and iodine. Thus it became possible to establish the requisite reference standard for the initial starch-iodine curve and the action of a known amount of enzyme on starch, respectively. A comparison of these curves is given in Fig. 1.

Curve A of Fig. 1 was prepared by making aqueous serial dilutions of 243 mg. of starch and 0.3 mg. of I_2 (in 15 mg. of KI). Curve B was prepared by incubating a constant amount of starch (243 mg.) with varying amounts of commercial alpha-amylase and treating the mixtures with the same volume of KI_3 as above. This second curve was the same whether water or heat-inactivated saliva was used as a diluent. From these data it was concluded that only the transmittance range from 18 to 80 percentile could be used because of the inflection points occurring below and above these values. Since a high degree of accuracy was not particularly necessary in our investigation, our

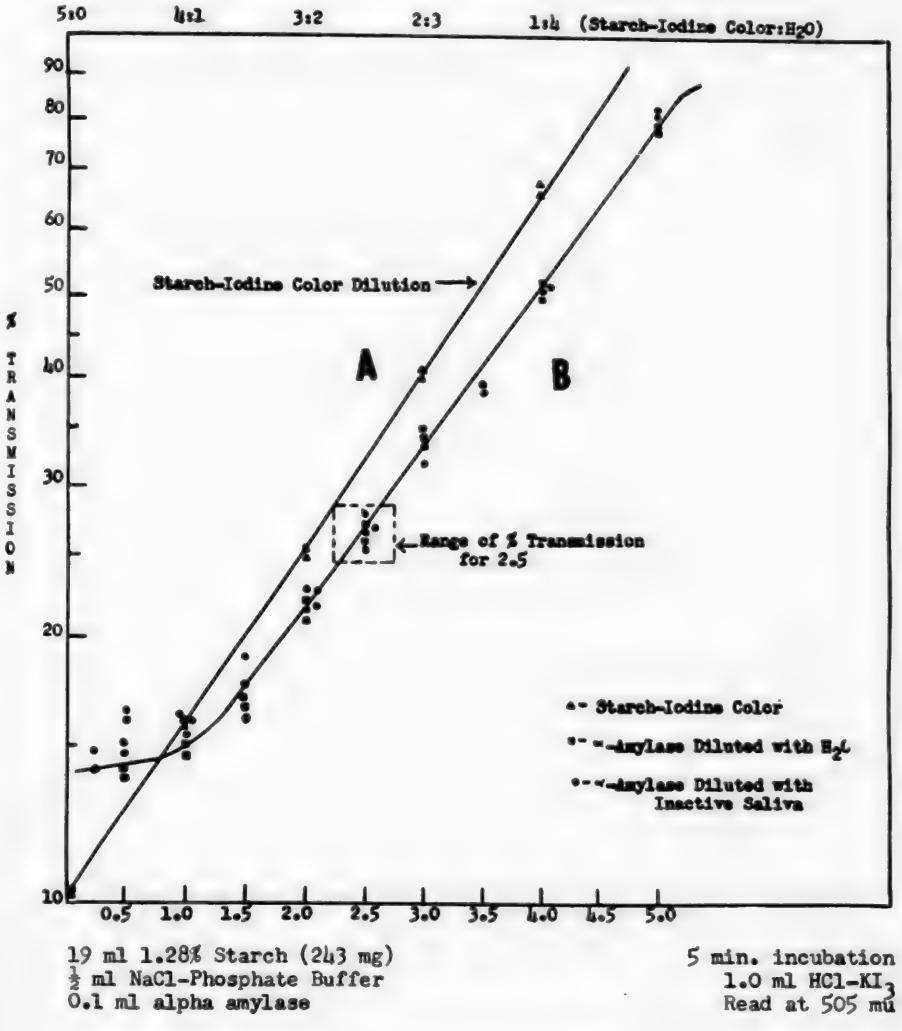
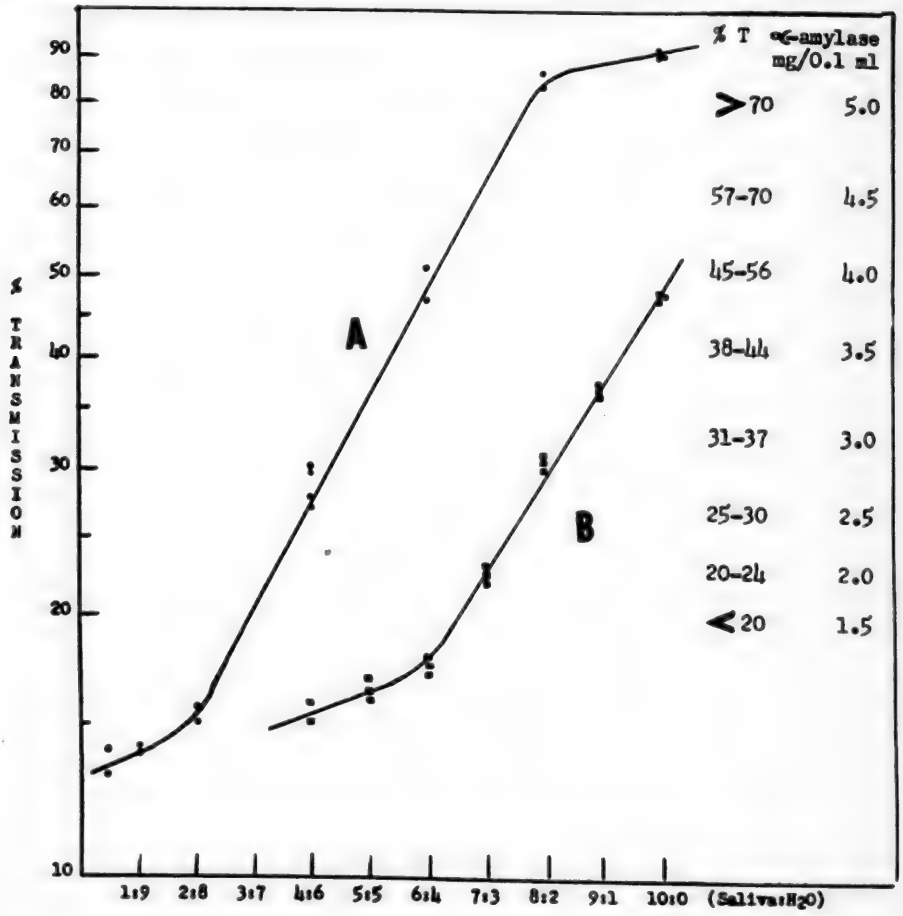


Fig. 1.—Starch-iodine color dilution.

standard reflected only increments of 0.5 mg. of amylase with a spread of ± 0.25 mg., e.g., any sample of saliva giving a transmission reading of 25 to 30 percentage was estimated to contain an equivalent of 2.5 mg. of commercial enzyme (see boxed area).



For explanation see text

Fig. 2.—Serial dilution of human salivas.

The validity of technic for measurement of the α-amylase content of human saliva was supported by the curve obtained when specimens were subjected to the same procedure as the control (starch α-amylase). The results are shown in Fig. 2.

This is a comparison of 2 specimens of human saliva, containing different amounts of alpha-amylase, which were treated the same as the standard using water as the diluent. The specimen containing a high concentration of the enzyme yielded a curve (A) with inflection points and linearity comparable to that of the standard (curve B, Fig. 1). The other curve (B) represents the data from a specimen of saliva with a lower content of alpha-amylase; it exhibited the same break at 18 percentile transmission but not at the upper point because of the smaller content of enzyme. The relative amounts of alpha-amylase reflected in the linear portion of the curves are given at the right side of the graph. These arbitrary units met the requirements of only the more active specimens of saliva. In our experience approximately 55 per cent of all samples gave less than 18 percentile transmission. However, an analysis of this group could be made by decreasing the amount of starch substrate used initially.

The details of our method as it was finally evolved and routinely applied are:

Saliva.—Specimens of saliva were collected from children at the Washington-Vancouver School for the Deaf between 9 and 10 A.M., placed as quickly as possible in an insulated box maintained just above freezing, and transported to the laboratory where they were placed under refrigeration (5° C.) until needed. All analyses (chemical and bacteriological) were completed within 24 hours. It has been shown by Bauer and Martin[11] and confirmed in our laboratory that under these conditions little change in activity occurs during this time. However, actual freezing of the specimens is to be avoided, since the amylolytic activity undergoes an apparent increase.

Reagents.—

1. *Starch Substrate, 1.28 per cent:* 12.80 Gm. of soluble indicator starch were accurately weighed and suspended in 100 ml. of cold water in a one-liter volumetric flask. To this was added 500 ml. of boiling water, with constant agitation to prevent clumping, then the flask was placed in boiling water until the solution steamed. After cooling to approximately 25° C., the contents were brought to volume with distilled water. This solution in 19 ml. aliquots was automatically buretted into 16 by 150 mm. test tubes. When stoppered, these can be stored at room temperature (20° to 25° C.) for 5 days without appreciable change. It is advisable to ascertain the reliability of the substrate prior to using by (1) checking the starch-iodine color relationship and (2) doing a standard amylase determination. Fresh starch should be prepared if these results show more than 3 per cent variation from those obtained the day the solution was prepared.

2. *NaCl-Phosphate Buffer, pH 7.0:* 0.2 Gm. of reagent grade NaCl was dissolved in 100 ml. of phosphate buffer (61.1 ml. of 0.1 M $Na_2HPO_4 \cdot 12\ H_2O$ and 38.9 ml. of 0.1 M KH_2PO_4). This mixture remains stable for one month at 5° C.

3. *Iodine Stock Reagent:* to 3 Gm. of reagent grade KI, dissolved in 90 ml. of water in a volumetric flask, 0.60 Gm. of resublimed I_2 was added and

shaken until dissolved, then diluted to volume with water. This solution is stable for at least 3 months at room temperature when stored in a glass-stoppered dark bottle.

4. *HCl-KI$_3$ Working Solution:* to 5 ml. of the Iodine Stock solution in a 100 ml. volumetric flask was added 25 ml. of 1.0 N HCl and water to dilute to volume. This must be prepared *fresh daily.*

5. *Alpha-Amylase Standard, 5 mg./0.1 ml.:* 0.5 Gm. of alpha-amylase (Nutritional Biochemical Corporation) was dissolved in 2 per cent aqueous NaCl to give a final volume of 10 ml. (5 mg./0.1 ml.). Aqueous serial dilutions were prepared from this stock solution to contain 4 mg., 3 mg., 2 mg., and 1 mg. of alpha-amylase per 0.1 ml. of the respective dilutions.

Procedure.—To the test tubes containing 19 ml. of starch substrate, 0.5 ml. NaCl-Phosphate buffer was added and the solution equilibrated in a water bath at 40° C. To each tube, 0.1 ml. saliva was added, mixed by inversion, and the whole returned immediately to the water bath. At the end of exactly 5 minutes' incubation, the enzymatic activity was stopped and the color developed by addition of 1.0 ml. of HCl-KI$_3$ working solution. The contents were mixed thoroughly by inverting several times and a portion read in a colorimeter (Bausch and Lomb Monochromatic, 505 mμ filter) set at 100 per cent transmission against a blank which contained 1.0 ml. of HCl-KI$_3$, 0.5 ml. of buffer, and 19 ml. of distilled water. To obtain reproducible results it was imperative that the transmittancy be read at a set interval (we used 30 seconds) after the addition of the last reagent. This was necessary because the development of the blue color is a function of time, increasing in intensity for 15 minutes and thereafter decreasing for 24 hours.[9]

RESULTS

After perfection this semiquantitative technic was introduced as a routine laboratory procedure for the determination of salivary alpha-amylase. Results on a distribution basis for 1,228 specimens of saliva are given in Table I.

TABLE I
THE DISTRIBUTION OF AMOUNTS OF ALPHA-AMYLASE IN SPECIMENS OF SALIVA

α-AMYLASE			
MG./0.1 ML. SALIVA	% T	NO. SPEC.	% OF NO.
1.5	20	665	54.7
2.0	20-24	185	15.1
2.5	25-30	129	9.9
3.0	31-37	72	5.9
3.5	38-45	54	4.4
4.0	46-56	44	3.6
4.5	57-70	19	1.5
5.0	70	60	4.9
		1,228	100.0

Analysis of Table I shows 55 per cent of the specimens had less than 1.5 mg. per milliliter of saliva of alpha-amylase while only 5 per cent had greater than 5.0 mg. per milliliter of saliva. Although it would be necessary to change

the amounts of starch to analyze the group at either extreme, this technic quickly and with reasonable precision separated individuals into 1 of 3 arbitrary groups based on the content of salivary alpha-amylase: high (5.0 mg. per milliliter), moderate (1.5 to 5.0 mg. per milliliter) and low (1.5 mg. per milliliter).

Using comparative data from these 1,228 samples we could find no relationship between alpha-amylase content and the lactobacillus count, acid production or volume of respective specimens of saliva.

DISCUSSION

The use of the starch-iodine color formation as an index of amylase attack upon starch requires a greater concentration of starch than does the technic of end products (reducing substances) analysis because at the erythrodextrin end point only 20 per cent of the starch is hydrolyzed and 80 per cent remains in the achromic region.[10] This relationship probably explained the inability to obtain the necessary linear standard of reference for photometric analysis when starch was the only variable; this was especially true in our case where large amounts of starch were required to provide enough substrate to offset the α-amylase content in 0.1 ml. of saliva. Although linearity of the starch-iodine mixture could be expected on the basis of simple dilution as we found it to be, we could not explain satisfactorily why a closely parallel linearity was also obtained when commercial amylase in progressive amounts (0.5, 1.0, 1.5, 2.0, etc.) acted upon the original and constant amount of starch. Nevertheless, the reactions gave transmission readings in conformance with the Beer-Lambert adsorption law and, as far as we could determine, were valid either for the approximation of known amounts of alpha-amylase in the standard or the unknown material.

We believe that this technic permits a quantitative estimation of alpha-amylase present in saliva because the short incubation time and high concentration of substrate create conditions under which the reaction rate should be dependent solely upon the enzyme concentration.[12] The term "amount" of enzyme is preferred to that of "activity" since a possibility exists that the activity of salivary alpha-amylase may be influenced by the presence of free amino acid of the dental plaque.[13, 14] It is extremely doubtful, however, that the concentration of these amino acids in the microamounts they must be in the saliva can materially affect the reaction rate in vitro tests requiring relatively huge amounts of substrate but it is a point which will bear further investigation.

SUMMARY

A rapid, quantitative method for estimation of salivary alpha-amylase has been developed. A known amount of starch is incubated with 0.1 ml. of saliva and the amount remaining is determined photocolorimetrically by development of the starch-iodine blue complex.

The amount of human salivary alpha-amylase present is determined by comparison with the action of commercial amylase under the same conditions.

Human salivas can be quickly grouped into 3 arbitrary groups in respect to their amylase content.

It is possible to prepare all the reagents, with the exception of the diluted working solution of iodine, at least 24 hours in advance of the time they are to be used.

Two technicians can analyze 150 samples of saliva per day.

There is no correlation between amounts of salivary alpha-amylase and lactobacillus counts, acid production in selective media or volume of respective specimens of saliva.

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THE EFFECTS OF PENICILLIN AND TERRAMYCIN ON DENTAL CARIES AND CERTAIN ORAL MICROFLORA IN HUNT-HOPPERT CARIES-SUSCEPTIBLE RATS

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CERTAIN antibiotics are able to inhibit dental caries presumably by their antimicrobial activity. Penicillin has proved to be of some value in reducing dental caries in humans[1, 2] and animals.[3-7] This antibiotic has also been shown to be effective in reducing numbers of aciduric bacteria in the mouth.[8-12] In animal experiments several investigators have shown a correlation between caries inhibition and lower numbers of oral lactobacilli when penicillin was incorporated into the diet.[5-7] Stephan, Fitzgerald, McClure, Harris and Jordan[7] showed that penicillin and bacitracin, which specifically inhibit gram-positive bacteria, are more effective in preventing dental caries than certain wide-spectrum antibiotics. However, since they found that Aureomycin did not inhibit lactobacilli, but controlled caries as well as Chloromycetin, which did inhibit lactobacilli, no conclusion could be made relating lactobacilli to dental caries.

The use of the Hunt-Hoppert caries-susceptible rats provides certain advantages over other strains. It is well known that this strain develops caries rapidly in contrast to the Hunt-Hoppert caries-resistant rats.[13-18] It has also been shown that lactobacilli are recovered more frequently and in larger numbers from caries-susceptible than from caries-resistant rats.[19-22]

Penicillin was selected for study because of its known ability to inhibit both caries and aciduric bacteria. Terramycin was chosen because it is effective against many types of bacteria, whereas penicillin chiefly inhibits gram-positive bacteria.

MATERIALS AND METHODS

Littermate caries-susceptible rats were distributed equally into test and control groups. A similar number of caries-resistant rats was also selected in order to compare the oral bacteriologic changes in antibiotic-inhibited caries with caries inhibited by heredity. Both male and female rats, 25 to 35 days old, were placed in cages containing wood shavings. The H. W. C. cariogenic

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diet was fed to the rats in the control group.[23, 24] The rats in the test groups were given the same diet containing, in addition, the following types and quantities of antibiotics:

Dibenzyldiethylenediamine penicillin (P 2.4)*	500 units per gram of feed
Terramycin animal formula soluble powder*	250 micrograms per gram of feed (0.025 per cent)

These antibiotic preparations were selected for their high stability in feeds. The concentration of Terramycin was determined by preliminary in vivo experiments which indicated the minimum concentration needed to inhibit lactobacilli. The above penicillin concentration was the same as used by previous investigators.[7]

Bacteriologic examinations of littermates were made on the same day at intervals of 1, 5, and 10 weeks. The lower right molars were brushed with a sterile cotton swab moistened in 1.0 per cent peptone water. The swab was retained in 1.0 ml. of the peptone water until the tip of the swab was broken off into a screw-cap bottle containing 5.0 ml. of 1.0 per cent peptone water. The bottle was shaken until the swab disintegrated. Dilutions were made in distilled water and 0.1 ml. amounts were plated on the surface of various agar media.

Lactobacillus counts were made on lactobacillus-selection medium (BBL) and on tomato juice agar special (Difco) containing 0.02 per cent sodium azide. Streptococcal counts were made on azide dextrose broth (Difco) containing 1.5 per cent agar and on brain heart infusion agar (Difco) containing 0.02 per cent sodium azide. Eosin methylene blue agar (Difco) was used for coliform counts. A "total" count was made on tryptone glucose extract agar (Difco) containing 4 per cent skim milk. All plates were incubated at 37° C. for 72 hours. Organisms per ml. of original suspension were used to determine the bacterial count.

The rats were observed macroscopically for caries every 2 weeks.

RESULTS

There was a marked difference in effectiveness between penicillin and Terraymycin in inhibiting dental caries. In Table I, it may be noted that Terramycin had retarded caries only slightly, whereas penicillin was extremely effective.

TABLE I
EFFECTS OF PENICILLIN AND TERRAMYCIN ON DENTAL CARIES

RAT STRAIN	ANTIBIOTIC IN DIET	NO. OF ANIMALS SURVIVING EXPERIMENT	PER CENT OF RATS WITH CARIES	AV. CARIES TIME (AGE OF RAT IN DAYS)
Susceptible	None	28	100	84
Susceptible	Penicillin	28	0	>200
Susceptible	Terramycin	27	100	109
Resistant	None	27	0	>200

*Antibiotics were supplied by Chas. Pfizer & Company, Inc., Terre Haute, Ind.

The bacteriologic findings are such that a definite association of any group of bacteria with the development of caries is unwarranted at this time. As seen in Table II, penicillin and Terramycin reduced oral lactobacillus counts with equal effectiveness. Higher numbers of streptococci were recovered from rats receiving Terramycin than from those receiving penicillin, especially when brain heart infusion agar was used. However, higher streptococcal counts were obtained from caries-resistant rats. No other differences in oral microflora could be detected between rats on the penicillin and Terramycin diet mixtures. Also, it may be noted that both test groups had slightly higher coliform counts and slightly lower total counts than their littermate controls.

TABLE II
THE EFFECTS OF PENICILLIN AND TERRAMYCIN ON CERTAIN ORAL MICROFLORA

RAT STRAIN	ANTIBIOTIC IN DIET	LACTOBACILLI		STREPTOCOCCI		COLIFORMS "TOTAL"	
		LBS*	TJA*	ADA*	BHI*	EMB*	TGE*
						(After one week)	
Susceptible	None	2,633	15,028	84,470	214,470	2,020	147,550
Susceptible	Penicillin	101	1,264	4,839	7,177	32,150	33,180
Susceptible	Terramycin	6	935	6,541	56,255	27,878	55,585
Resistant	None	476	1,914	33,805	37,125	9,994	49,966
						(After five weeks)	
Susceptible	None	1,252	11,505	127,570	147,346	7,508	154,767
Susceptible	Penicillin	193	3,046	7,600	10,577	25,967	51,633
Susceptible	Terramycin	100	4,790	18,336	65,154	29,857	80,714
Resistant	None	317	642	146,038	182,076	10,035	244,615
						(After ten weeks)	
Susceptible	None	2,324	39,286	244,758	368,808	12,586	320,828
Susceptible	Penicillin	177	2,210	7,120	7,569	29,437	40,267
Susceptible	Terramycin	246	9,136	24,275	80,959	33,204	98,625
Resistant	None	137	12,659	130,720	198,400	15,980	219,080

*LBS = Lactobacillus-selection medium; TJA = Tomato juice agar; ADA = Azide dextrose agar; BHI = Brain heart infusion agar; EMB = Eosin methylene blue agar; TGE = Tryptone glucose extract agar.

Although no attempt was made to identify bacteria, it was noted that a different type of colony could be recognized on tomato juice agar among rats receiving antibiotics. Instead of the usual flat, translucent colony with an irregular edge, most of the colonies (between 90 and 100 per cent) tended to be raised, opaque, and with an entire edge. Upon microscopic examination, these proved to be cocci indicating that a streptococcal rather than a lactobacillus count was obtained on tomato juice agar from the rats receiving antibiotics. However, since the use of both penicillin and Terramycin resulted in the same change in the oral flora, this point was not further investigated.

DISCUSSION

The results obtained when penicillin was incorporated into the diet parallel those of other investigators. The results obtained with Terramycin are somewhat perplexing. This antibiotic inhibited lactobacilli to the same degree as penicillin, and streptococci almost as well as penicillin, yet caries developed in all of the rats receiving Terramycin with almost the same rapidity as in control rats.

A logical explanation for these results might be that an organism involved in the etiology of dental caries is inhibited by penicillin but not by Terramycin. This cryptic organism would require media other than those used in this study or different technics of cultivation for its detection. At best, only a small fraction of the oral microflora can be considered by one group of investigators during a single study.

These results do not necessarily mean that lactobacilli and other acidogenic bacteria are no longer of importance as possible etiologic agents in dental caries. Lactobacilli have been recovered consistently in larger numbers and with greater frequency from caries-susceptible than from caries-resistant rats.[19-22] However, when resistant rats develop caries, the percentage of rats from which lactobacilli are recovered is significantly lower than in susceptible rats.[22] In this study, rats receiving Terramycin also developed caries but failed to show as high numbers of lactobacilli as in the control group. Therefore, it is possible that the microorganism or microorganisms responsible for caries depends on the nature of resistance offered by the animal. It is conceivable that lactobacilli may be responsible for caries in the susceptible rats when maintained on the unaltered cariogenic H. W. C. diet. When Terramycin is added to this diet, another group of organisms may initiate caries. The same or another group of organisms may be the causative agent of dental caries in the resistant rats.

SUMMARY

Penicillin at a concentration of 500 units per gram of feed effectively inhibited dental caries and several groups of acidogenic bacteria usually associated with dental caries. In contrast, Terramycin at a concentration of 0.025 per cent in the feed retarded dental caries only slightly although the bacteriologic findings were quite similar to those in the penicillin group. These results suggest that organisms other than those considered in this study may play an essential role in the etiology of dental caries in these animals.

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STUDIES ON THE ACIDURIC ORAL MICROCOCCI

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INTRODUCTION

A NUMBER of investigators have considered the oral micrococci from the standpoint of the possibility of their involvement in the production of dental caries.[1, 2] The ability of some of the oral micrococci to produce and survive in substantial amounts of acid in carbohydrate media was pointed out by Florestano.[3] Stephan and Hemmens[4] and Stralfors[5] have presented quantitative data on the rate and extent of acid production from carbohydrates by certain oral micrococci. Morris[6] concluded that the staphylococci occur more frequently in the mouths of caries-susceptible individuals than in the mouths of caries-free individuals and that lactose-fermenting micrococci predominate in the former. Some attention has also been directed toward the possible pathogenicity of these organisms. Singer[7] has attempted to correlate physiological characteristics and toxigenic properties of staphylococci isolated from saliva. Using a selective medium, Singer was able to isolate staphylococci from the salivas of 52 of 53 individuals sampled.

In the current edition of *Bergey's Manual*[8] all gram-positive, catalase-positive, aerobic cocci are included in the genus *Micrococcus*. However, the term *Staphylococcus* is still commonly used, especially among clinical bacteriologists, in referring to those micrococci having the characteristics of the former genus *Staphylococcus*. Such organisms form irregular, grapelike clusters of cells, grow best at body temperature, and may be actual or potential pathogens. It is in this specialized sense that the term "staphylococcus" is used in this paper.

During the performance of routine salivary lactobacillus counts, the growth of micrococci on tomato juice agar plates at pH 5.0 has been commonly observed. Since the ability to survive and grow at this pH level seemed to distinguish such strains from the pathogenic and saprophytic micrococci usually encountered in nature, the present study was undertaken in order to gain additional information on the aciduric oral micrococci.

EXPERIMENTAL METHODS

Paraffin-stimulated saliva samples were collected from 59 adults in mid-morning at least 2 hours after breakfast. One-tenth milliliter of a 1:5 dilution of the salivas in broth was streaked on the surface of pH 5.0 tomato juice

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agar plates, which were then incubated aerobically at 37° C. for 4 days. On this medium micrococcus colonies were generally 2 to 4 mm. in diameter, of a creamy consistency, raised, and with an entire margin. The surface texture varied considerably. Some colonies were smooth and dull, others were finely pitted, and still others presented a mulberry-like appearance. Pigmentation, when present, ranged from tan to golden.

Colonies of gram-positive, catalase-positive cocci[9] were purified by streaking on mannitol salt agar.[10] The pure strains were stored in the refrigerator in stab cultures of trypticase-soy agar (BBL) and transferred monthly. An additional set was stored at –20° C.

Pigmentation (ranging from tan to golden) and gelatinase production were determined on Staphylococcus medium 110.[11]

Hemolysin production was tested in pour plates of Difco brain heart infusion (BHI) agar containing 5 per cent defibrinated sheep blood. The blood plates were incubated at 37° C. for 36 hours and refrigerated overnight before examination for hemolysis. A repetition of this test using rabbit blood gave essentially the same results.

Fermentation reactions were determined in tubes of phenol red broth base containing the individual carbohydrates and Durham tubes to detect possible gas production. Carbohydrates were sterilized routinely by filtration and added aseptically to a final concentration of 1 per cent; inulin was the sole exception, being autoclaved in the medium for 10 minutes at 110° C. The temperature range for optimum growth was determined by incubating BHI slants of the strains in air at 20°, 30°, 37°, and 45° C.

Coagulase tests were performed on both fresh human plasma and Bacto-Coagulase plasma with comparable results.

All strains were screened for phosphatase activity at pH 6.0 and pH 8.7, with disodium p-nitrophenyl phosphate (Sigma) as the substrate, following the procedure employed by Fitzgerald.[12]

Other tests performed included nitrate reduction, indole and H_2S production, urease activity, and utilization of urea or $NH_4H_2PO_4$ as sole source of nitrogen.

The inocula for all tests were derived from 24-hour trypticase-soy (BBL) broth cultures. Suitable controls were included in all cases.

RESULTS

A total of 109 strains of gram-positive, catalase-positive aerobic cocci were isolated from salivas of 46 of the 59 individuals sampled. On the basis of biochemical and morphological characteristics the aciduric oral micrococci were separated into 3 groups as shown in Table I.

Seventeen coagulase-positive strains of staphylococci isolated from 14 individuals have been placed in Group I. All of the strains in this group fermented mannitol, hydrolyzed gelatin, and reduced nitrate to nitrite. Sixteen of the 17 strains were pigmented and 13 hemolysed rabbit or sheep red blood

cells. All possessed phosphatase activity at pH 6.0 but not at pH 8.7. Stained smears revealed gram-positive, spherical cocci, 0.8-1.0 μ in diameter arranged in typical staphylococcal clusters.

Eighty strains of micrococci, isolated from 52 individuals, were morphologically similar to those of Group I but did not produce coagulase or, with one exception, golden yellow pigment. Since they also displayed individual differences in response to the other biochemical tests, they have been placed together in Group II to form a miscellaneous group of "nonpathogenic" oral micrococci.

TABLE I
CHARACTERISTICS OF ACIDURIC MICROCOCCI ISOLATED FROM THE HUMAN MOUTH

	GROUP I (17 STRAINS) NO. POSITIVE	GROUP II (80 STRAINS) NO. POSITIVE	GROUP III (12 STRAINS) NO. POSITIVE
Coagulase	17	0	0
Gold pigment	16	1	0
Mannitol	17	6	1
Salicin	8	1	0
Fructose	17	80	9
Acid phosphatase (pH 6.0)	17	73	0
Alkaline phosphatase (pH 8.7)	0	0	0
Gelatinase	17	67	0
Nitrate reduction	17	67	11
V-P reaction	0	44	0
Urease	5	67	11
Hemolysis	13	61	3
Catalase	17	80	12
Growth at 45° C.	0	0	0
Growth at 20° C.	scanty	scanty	scanty
Opt. growth range	30-37° C.	30-37° C.	30-37° C.
Av. final pH glucose (7 days)	4.3	4.3	4.4
Av. final pH sucrose (7 days)	4.3	4.3	4.8

All fermented: glucose, maltose, glycerol, sucrose.
None fermented: arabinose, xylose, raffinose, inulin.
All strains negative in: indole production, starch hydrolysis, H_2S production, growth with $NH_4H_2PO_4$ or urea as sole nitrogen source.

The 12 strains which comprised Group III were isolated from 10 individuals and could be distinguished from those of the first 2 groups on the basis of microscopic appearance of gram-stained smears of broth cultures. The cells of this group were typically arranged in tetrads with the adjacent sides of paired cells flattened. In addition, the size of the individual cells averaged about 1.5 to 1.7 μ which is larger than the cells of Groups I and II (0.8-1.0 μ). Although the tetrad arrangement is characteristic of the genus *Gaffkya*, the organisms in Group III differ from *G. tetragena*, the single aerobic member of the genus recognized in *Bergey's Manual*,[8] by the fact that 11 of the 12 oral strains reduced nitrate to nitrite, a property not possessed by *G. tetragena*.[8, 13] None of the strains in Group III produced coagulase, acid phosphatase, or gelatinase. The fermentative powers of the Group III organisms in glucose and sucrose were not as pronounced as those in the other groups. For example, the strains of Group I and Group II attained their maximal acidity (pH 4.3) from glucose or sucrose within 48 hours whereas the strains of Group III attained an acidity of pH 4.7 from glucose and pH 5.7 from sucrose in this time.

After 6 months' storage in trypticase-soy stabs at –20° C., a number of tests were repeated in order to check the biochemical variability of micrococci which has been commonly encountered.[14-16] Forty-eight strains selected as being representative members of the three groups were tested. Table II shows that 19 of the 48 strains gained or lost the ability to carry out a given reaction. It can be seen that variability was not confined to any particular group nor was there any distinguishing pattern in the changes which occurred.

TABLE II
VARIABILITY OF BIOCHEMICAL CHARACTERISTICS IN ORAL MICROCOCCI

	NEGATIVE TO POSITIVE			POSITIVE TO NEGATIVE		
	GROUP			GROUP		
PROPERTY	I 8 STRAINS STRAIN #	II 30 STRAINS STRAIN #	III 10 STRAINS STRAIN #	I 8 STRAINS STRAIN #	II 30 STRAINS STRAIN #	III 10 STRAINS STRAIN #
Fermentation of:						
Fructose	—	—	71, 18	—	—	—
Lactose	—	6, 109	—	—	—	—
Mannitol	—	89	—	—	7, 8	—
Salicin	10	—	71	49	81, 96	—
Production of:						
Pigment	49	64, 109, 89 110	—	55	—	—
Gelatinase	—	—	—	—	4, 38, 81 28, 64	—
Urease	55	—	—	—	6, 115, 38	—
Acid phosphatase	—	—	—	—	—	—
Acid in litmus milk	—	107	—	—	89	—
Reduction of nitrate	—	—	—	—	—	—

Total strains tested: 48. Numbers of strains showing variation: 19.

TABLE III
ACID PHOSPHATASE ACTIVITY OF ORAL MICROCOCCI

	GROUP I (8 STRAINS)		GROUP II (28 STRAINS)	
pH	AVERAGE	RANGE	AVERAGE	RANGE
3.5	.164*	(.097-.320*)	.024	(.000-.071)
4.5	.525	(.415-.714)	.328	(.044-.783)
5.1	.831	(.763-.899)	.658	(.024-.885)
5.7	.753	(.672-.831)	.678	(.239-.849)
6.1	.768	(.675-.855)	.737	(.325-.875)
6.5	.749	(.622-.823)	.627	(.229-.813)
6.8	.752	(.637-.859)	.573	(.253-.773)
7.4	.398	(.160-.685)	.334	(.065-.464)

*Corrected density at 430 mμ.

Conditions: Inoculum was the centrifuged and washed cells of 24-hour cultures in 5 ml. trypticase-soy broth; fresh cultures were used at each pH level. Substrate was 0.4 mg. disodium p-nitrophenyl phospate in 3.0 ml. of 0.1 molar maleate buffer; incubation time 60 minutes at 37° C.

In order to obtain further information concerning the characteristics of the phosphatase activity of the aciduric micrococci 8 strains of Group I and 28 strains of Group II were tested for phosphatase activity over the pH range 3.5 to 7.4. A fresh 24-hour culture grown in 5 ml. trypticase-soy broth was used as inoculum for the test at each pH level. The organisms were centrifuged, washed once with saline, and tested by the method of Fitzgerald,[12] in maleate

buffer of the appropriate pH. The results are shown in Table III as the average, and range of, activity for representative strains of Group I and II at each pH level. Since a fresh culture was used at each pH level, strict comparisons are not justified; however, it is evident that the average acid phosphatase activity varies with pH in a similar manner for each group. In addition the range of variation within groups at each pH level as shown in Table III makes it apparent that a separation of the oral micrococci of Group I and Group II strains solely on the basis of phosphatase activity is not feasible.

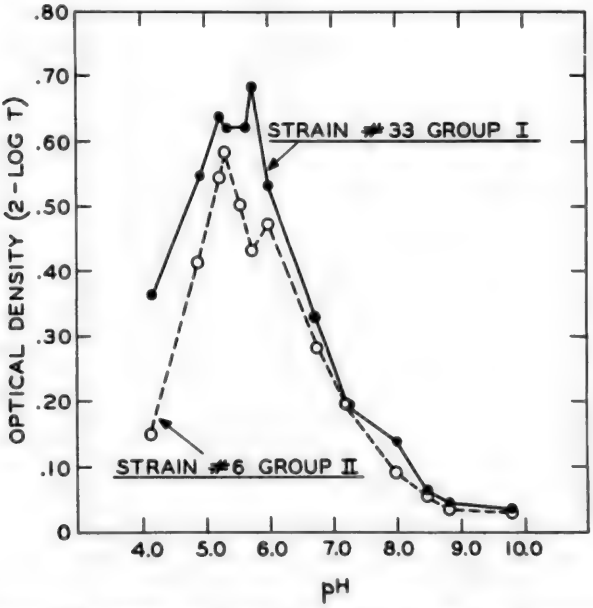

Fig. 1.—Variation of phosphatase activity with pH in two oral micrococcus strains.

Shown in Fig. 1 are pH-activity curves for a single strain each of Group I and Group II. Mass cultures of each of the 2 strains were grown for 24 hours in 100 ml. trypticase-soy broth. After centrifugation and washing in saline the cells were made into a 1 per cent suspension by volume. Five-tenths milliliter of this suspension was used as inoculum at each pH level tested. As can be seen from Fig. 1 and Table III, the enzymatic activity of both groups was most pronounced at pH 5.0 to 6.0. The strains of Group I appeared to exhibit greater phosphatase activity throughout the range tested; however, there was sufficient variation with pH (Table III and Fig. 1) to indicate the possibility that more than a single phosphatase was present in these organisms. The most pronounced difference between the 2 groups was seen at the lower end of the pH scale where the average activity of the Group I strains was greater than that of the Group II strains.

DISCUSSION

A number of different types of micrococci can be isolated from human saliva on pH 5.0 tomato juice agar. Some of these strains exhibit certain physiologic characteristics in common with "pathogenic" micrococci such as production of hemolysin, fermentation of mannitol, and coagulase and phosphatase activity.[17, 18] On the basis of the current classification in *Bergey's Manual*[5] these aciduric strains (Group I) would be considered as *Micrococcus pyogenes, var albus* or *var. aureus,* depending on pigmentation. At least 2 other groups of oral micrococci may be differentiated from those of Group I on the basis of lack of pigmentation and inability to coagulate plasma. The members of Groups II and III show little homogeneity in respect to their comparative physiological characteristics and cannot be identified by the use of any of the available classification schemes. They have been separated from each other principally on the basis of microscopic morphology and arrangement of the cells. The strains in Group II like those of Group I were arranged in typical staphylococcal clusters of spherical cells while those in Group III were characteristically arranged in tetrads in which the adjoining surfaces of the cells were flattened. None of the members of Group III fully conform to the description of the single aerobic species in the genus *Gaffkya, G. tetragena,* recognized in *Bergey's Manual,* the main difference being the ability to reduce nitrates. All of the Group III strains are strongly catalase positive even in acid media and this together with the fact that the pediococci do not reduce nitrates would also seem to exclude them from the genus *Pediococcus* as presently defined.[19, 20]

Studies on the comparative phosphatase activity of the oral micrococci assume some additional significance in view of the recent interest in the use of phosphatase tests as aids in identifying "pathogenic" staphylococci. Using phenolphthalein disphosphate as a substrate for cultures growing at pH 7.4-7.6, Barber[17] found that coagulase-positive staphylococci were phosphatase-positive within 18 to 24 hours while coagulase-negative strains, if able to do so at all, required 48 hours or longer to hydrolyze the substrate. White and Pickett,[18] using nonproliferating suspensions of *Micrococcus pyogenes* in 0.01 M citrate buffered at pH 5.6, showed that 20 coagulase-positive strains hydrolyzed disodium phenyl phosphate within a 4-hour incubation period at 37° C., while 13 coagulase-negative strains failed to do so. On this basis these workers suggested the use of the phosphatase test as a substitute for the coagulase test for detection of potentially pathogenic strains. With the oral micrococci tested in the present study such a bilateral correlation was not found. While all coagulase-positive oral micrococci also showed acid-phosphatase activity, the converse was not true as 73 of the 80 coagulase-negative strains of Group II were phosphatase-positive (Table I). When all of the oral micrococci were retested for phosphatase activity at pH 6.0 using phenolphthalein diphosphate (Sigma) in maleate buffer, the results were identical with those obtained with disodium p-nitrophenyl phosphate (Sigma).

In a second experiment simultaneous phosphatase tests were run on 28 representative oral micrococcus strains to compare the procedure of White and

Pickett[18] (using disodium phenyl phosphate in citrate buffer at pH 5.6) and our own method (using disodium p-nitrophenyl phosphate in maleate buffer at pH 5.6). Again the results were identical except for the fact that in the method of White and Pickett an incubation period of 4 hours at 37° C. was required to demonstrate activity, whereas, when disodium p-nitrophenyl phosphate was used, positive phosphatase activity was demonstrated in as little as 30 minutes' incubation at 37° C. Both coagulase-positive and coagulase-negative oral micrococci were used in this test and all of the coagulase-positive strains were positive by either method, while some coagulase-negative strains were positive and some negative for phosphatase activity. As indicated above, however, both methods gave identical results. This would imply, at least as far as the oral micrococci are concerned, that the phosphatase test alone is not a reliable substitute for the coagulase test for "pathogenic" micrococci.

In view of the feeling of some investigators[21, 22] that staphylococci are rapidly becoming the most important and most common cause of bacterial infections, it becomes of some importance to attempt to determine their prevalence and their possible significance in the oral cavity. Singer[7] has already discussed some of the characteristics of the oral staphylococci which would make them potentially important as primary invaders or contributory agents in diverse pathological conditions in the oral cavity. Included among these are the elaboration of toxins, predilection for developing focal infections, the frequency with which these organisms occur in osteomyelitis and root canal infections, and their ability to produce acid, hydrolyze proteins, and coagulate plasma. Added to these considerations is the readiness with which the micrococci develop resistance to antibiotic agents.[21-23]

TABLE IV
FREQUENCY OF ISOLATION OF POTENTIALLY PATHOGENIC MICROCOCCI FROM SALIVA IN TWO INDEPENDENT STUDIES

	SINGER (1952)	PRESENT STUDY
Isolation medium	Staph. Medium #110	Tomato Juice Agar (pH 5.0)
No. individuals sampled	53	59
Samples positive for staphylococci	52	46*
Total strains isolated	89	97*
Per cent isolates positive for:		
Golden pigment	47%	16%
Coagulase	18%	18%
Gelatinase	74%	87%
Hemolysin	50%	76%
Mannitol fermentation	11%	24%
Pathogenic† types:		
Per cent of isolates	3%	16%
No. of individuals yielding pathogenic types	3 (6%)	14 (24%)

*Refers to strains with the morphological characteristics of staphylococci.

†According to Evans,[24] strains which produce golden pigment, coagulate serum, hydrolyze gelatin, and ferment mannitol are considered "pathogenic."

Singer,[7] using a selective medium (*Staphylococcus* medium #110, Difco) isolated staphylococci from the saliva of 52 of 53 individuals sampled. In the

present study staphylococci were isolated on pH 5.0 tomato juice agar plates from salivas of 46 of the 59 individuals sampled.

Table IV presents a comparison of Singer's findings with our results in terms of the frequency with which potentially pathogenic staphylococci were isolated. The criteria used for "pathogenicity" are those of Chapman[16] and Evans,[24] namely, those strains which produce golden pigment, are coagulase-positive, liquefy gelatin, and ferment mannitol. The data indicate coagulase-positive micrococci were found with equal frequency (18 per cent) in each study, while strains with all of the above physiological characteristics of "pathogenic" staphylococci were encountered with greater frequency in the present study (16 per cent compared to 3 per cent). Inasmuch as experimental conditions in the 2 studies were not strictly comparable, no special significance is attached to this difference. It is clear, however, that at least some of the potentially pathogenic micrococci are sufficiently aciduric in character to survive and proliferate at a pH level (pH 5.0) at which most pathogenic bacteria neither grow nor survive. The fact that these microorganisms were present in the saliva of 24 per cent of the cases in the present series suggests that the oral cavity may serve as a reservoir as well as a route of transmission of the staphylococci. If this surmise is true, the oral micrococci must be considered not only from the standpoint of oral infections but also in the broader aspects of their general public health significance. Considered in this light, one of the obvious implications would be the possibility of contributing to the development and spread of antibiotic-resistant *Micrococcus pyogenes* strains by indiscriminate use of antibiotics, locally or systemically, in patients who may harbor potentially pathogenic types in their oral cavity.

SUMMARY

A total of 109 strains of aerobic, gram-positive, catalase-positive micrococci were isolated from tomato juice agar (pH 5.0) plates from the salivas of 46 of 59 adults sampled. A study of the biochemical characteristics of these strains revealed that 16 strains from 14 individuals were typical *Micrococcus pyogenes var. aureus (Staph. aureus)* and one strain was identified as *M. pyogenes var. albus (Staph. albus)*. All of these strains (Group I) were coagulase-positive, gelatinase-positive, and fermented mannitol. The remaining strains consisted of a group of 80 coagulase-negative micrococci (Group II) and a group of 12 strains of phosphatase-negative, tetrad-forming micrococci (Group III).

All of the coagulase-positive oral micrococci (Group I) showed acid phosphatase activity with disodium p-nitrophenyl phosphate, phenolphthalein phosphate, or phenyl phosphate as substrates. Most of the coagulase-negative oral micrococci of Group II also possessed similar acid phosphatase activity for these 3 substrates, which would indicate that the phosphatase test alone is not a reliable substitute for the coagulase test. None of the micrococci showed alkaline phosphatase activity at pH 8.7.

Part of this material was taken from the thesis of H. V. Jordan, submitted to the Department of Bacteriology, University of Maryland, College Park, Maryland, June, 1952, in partial fulfillment of the requirements for the Master of Science degree.

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INFLUENCE OF FLUORIDES ON THE RATE OF DISSOLUTION OF THE PRINCIPAL INORGANIC ELEMENTS OF DENTIN

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THE exposure of dentin and enamel to fluorides decreases their solubility in acids, with some fluorides being more active than others.[1-4] The solubility of the tooth tissues usually has been determined gravimetrically[1, 4, 5] but this method does not measure the loss of the individual inorganic tissue components and is not entirely satisfactory for determining the solubility of tooth tissues whose weight is increased when they are exposed to certain fluorides.[1, 6, 7] Muhler, Boyd, and Van Huysen[7] determined the acid solubility of treated and untreated powdered tooth tissues by measuring the amount of calcium and phosphorus released during short periods of decalcification with acetate buffers. In such studies the total amount of soluble calcium and phosphorus differed between control and treated tissues. The calcium:phosphorus ratio also differed between treated tissues and between treated and control tissues, indicating that fluorides may influence the rate of dissolution of the individual inorganic elements of dental tissues as well as affecting the solubility of their entire apatite moiety. It seemed likely, therefore, that further investigation of the rate of dissolution of calcium, phosphorus, magnesium, and carbon dioxide of untreated and fluoride-treated dentin may indicate whether the elements were influenced individually or whether the solubility of the entire inorganic molecular entity was affected by the fluoride ions, and thereby give some additional knowledge of the action of fluorides upon dentin. This investigation is concerned, therefore, with the rate of dissolution by acids of calcium, phosphorus, magnesium, and carbon dioxide from untreated and fluoride-treated dentin.

METHODS

Twelve sections of dentin, weighing approximately 100 mg. each, were obtained from 3 extracted teeth whose history was known. The sections were intermingled into 3 equal groups and dried to a constant weight at 100° C. in vacuo over calcium chloride. The sections in 1 group were immersed in distilled water for 6 days, while those in the other 2 groups were immersed in 2 per cent sodium fluoride and 2 per cent calcium fluoride, respectively, for 6 days. After treatment, the dentin sections were thoroughly washed in distilled water and again dried to a constant weight at 100° C. in vacuo over calcium chloride. Each of the sections of the 3 groups were then decalcified separately for 8 hourly intervals in 3.2 ml. of Morse solution[8] in a Warburg apparatus at

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37° C. and the carbon dioxide produced was measured by standard manometric technics.[9] The partially decalcified dentin sections were dried in vacuo at 100° C. for 24 hours between the hourly immersions in Morse solution, thereby establishing the weight loss of each of the 8 decalcification periods. When a decalcifying solution was analyzed just as it was obtained from the Warburg flask, wide variations in phosphorus were found in different aliquots taken from it, and magnesium could not be detected, presumably because of the interference of organic material in the solution. To correct this, prior to analysis each decalcifying solution was oxidized with nitric acid while reducing it to dryness. The dry, oxidized sample was then dissolved in 0.1N HCl, from which aliquots were taken for analysis. There was no reduction in the amount of phosphorus or magnesium by this treatment and no interference with the analysis of these elements. The solutions of the oxidized samples for the hourly intervals were analyzed for calcium, phosphorus, and magnesium by the methods of Sobel and Sklersky,[10] Sobel,[11] and Orange and Rhein,[12] respectively, with slight modifications. Since the solution to be analyzed contained no organic material, trichloroacetic acid precipitation was omitted wherever used in the original methods.

RESULTS

The rates of dissolution of the principal inorganic components were first determined at hourly intervals for water-treated dentin (Table I). The amount

TABLE I
THE RATE OF DISSOLUTION OF THE PRINCIPAL INORGANIC CONSTITUENTS OF WATER-TREATED DENTIN

HOURS OF DECALCIFICATION	% WEIGHT LOST	DENTIN* % Ca	% P	% Mg.	% CO_2	% ELEMENTS RECOVERED
1	18.5	34.8	17.3	1.25	2.84	56.0
2	12.5	31.9	12.4	1.42	3.40	57.2
3	9.9	30.8	15.5	1.47	3.34	53.2
4	5.9	36.0	17.1	1.51	5.01	64.8
5	7.2	36.8	17.5	1.24	4.07	39.7
6	5.3	35.7	17.1	1.55	2.78	67.7
7	7.8	34.6	17.2	1.39	3.18	59.5
8	10.2	37.5	17.3	2.01	4.14	62.7
Mean and Standard Error	9.7 ± 1.5	34.8 ± 0.82	16.4 ± 0.61	1.48 ± 0.08	3.60 ± 0.27	57.6 ± 3.06

*Decalcifying agent: Morse solution.

of the inorganic elements released during each hour of decalcification is expressed as a percentage of the total weight loss. The mean percentage weight loss of the 4 dentin sections during the 8 hours of decalcification by Morse solution was 9.7 ± 1.5 per cent, with a tendency for more of the tooth tissue to dissolve at the beginning of decalcification than during later stages. The rate of dissolution of calcium, phosphorus, magnesium, and carbon dioxide did not change significantly as decalcification progressed. Calcium, phosphorus, magnesium, and carbon dioxide averaged 34.8 ± 0.82 per cent, 16.4 ± 0.61 per cent, 1.48 ± 0.08 per cent, and 3.60 ± 0.27 per cent, respectively, of the total weight

lost during 8 hours of decalcification. The total of the inorganic elements recovered by analysis accounted for 57.6 ± 3.06 per cent of the weight lost during decalcification.

TABLE II
THE RATIOS OF VARIOUS ELEMENTS RELEASED FROM WATER-TREATED DENTIN DURING DECALCIFICATION WITH MORSE SOLUTION

HOURS OF DECALCIFICATION	Ca:P	Ca:Mg.	Ca:CO_2	P:Mg.	Mg.:CO_2
1	1.98	27.8	12.2	13.8	0.44
2	2.43	22.5	9.3	8.7	0.41
3	1.99	20.9	9.2	10.5	0.44
4	2.03	23.8	7.1	11.3	0.30
5	2.56	29.6	9.0	14.1	0.30
6	2.18	23.0	12.8	11.0	0.55
7	2.08	24.9	10.8	12.4	0.43
8	1.94	18.6	9.0	8.6	0.48
Mean and Standard Error	2.14 ± 0.08	23.8 ± 1.26	9.9 ± 0.66	11.3 ± 0.73	0.41 ± 0.03

The ratios of the various elements released from water-treated dentin at hourly intervals of decalcification are shown in Table II. The calcium:phosphorus ratios fluctuated only slightly during the entire period of decalcification, averaging 2.14 ± 0.08. The calcium:magnesium ratios were highest, averaging 23.8 ± 1.26; the calcium:carbon dioxide ratios averaged 9.9 ± 0.66; the average of the phosphorus:magnesium ratios was 11.3 ± 0.73; the average of the magnesium:carbon dioxide ratios was 0.41 ± 0.03.

TABLE III
THE RATE OF DISSOLUTION OF THE PRINCIPAL INORGANIC CONSTITUENTS OF CALCIUM FLUORIDE-TREATED DENTIN*

HOURS OF DECALCIFICATION	% WEIGHT LOST	% Ca	% P	% Mg.	% CO_2	% ELEMENTS RECOVERED
1	22.1	43.8	11.2	0.8	4.2	59.9
2	14.5	40.2	14.4	1.4	1.4	57.4
3	7.3	34.3	17.4	1.5	2.4	55.6
4	4.2	35.0	16.7	2.6	—	54.9
5	4.8	36.2	17.6	1.5	2.7	56.8
6	3.5	34.4	17.5	1.2	1.9	54.1
7	4.2	33.8	17.0	1.8	1.6	54.2
8	1.5	35.0	17.4	1.5	1.3	55.4
Mean and Standard Error	7.8 ± 2.5	36.6 ± 1.25	16.2 ± 0.79	1.5 ± 0.18	2.2 ± 0.38	56.0 ± 0.69

*Decalcifying agent: Morse solution.
(–) = No value obtained.

The rates of dissolution of the principal inorganic constituents of calcium fluoride-treated dentin are shown in Table III. The average percentage weight lost during the 8 hours of decalcification was 7.8 ± 2.5, which was slightly less than was lost from the water-treated control dentin, decalcified similarly. Calcium fluoride-treated dentin lost more weight during the early stages of decalcification than it did during the later stages. More calcium was released during the first stages of decalcification than was released during the later stages but the rate of release of phosphorus was somewhat reversed. There was

no consistent change in the rate of release of magnesium or carbon dioxide from calcium fluoride-treated dentin. The total of the inorganic elements recovered from calcium fluoride-treated dentin accounted for 56.0 ± 0.69 per cent of the weight lost during decalcification.

TABLE IV
THE RATIOS OF VARIOUS ELEMENTS RELEASED FROM CALCIUM FLUORIDE-TREATED DENTIN DURING DECALCIFICATION WITH MORSE SOLUTION

HOURS OF DECALCIFICATION	Ca:P	Ca:Mg.	Ca:CO_2	P:Mg.	Mg.:CO_2
1	3.39	55.4	10.4	14.1	0.18
2	2.78	29.5	29.3	10.6	0.99
3	1.64	23.1	14.4	11.7	0.62
4	1.95	13.2	—	6.3	—
5	2.00	24.6	13.5	11.9	0.54
6	2.12	27.9	18.1	14.2	0.65
7	2.01	18.6	21.1	9.4	1.13
8	1.99	23.6	26.3	11.7	1.11
Mean and Standard Error	2.23 ± 0.20	26.9 ± 4.43	19.0 ± 2.62	11.2 ± 0.90	0.74 ± 0.13

(–) = No value obtained.

The ratios of the various elements released from calcium fluoride-treated dentin at hourly intervals of decalcification are shown in Table IV. There was more variation in the ratios of the different elements released from calcium fluoride-treated dentin than there was for the elements released from water-treated dentin. Also, the ratios between calcium and phosphorus or calcium and magnesium were relatively higher during the first few hours of decalcification than they were during the later decalcification periods, indicating that an increased amount of calcium was released at the beginning of decalcification. The calcium:carbon dioxide ratios for the calcium fluoride-treated dentin were consistently greater than those of the water-treated control dentin.

TABLE V
THE RATE OF DISSOLUTION OF THE PRINCIPAL INORGANIC CONSTITUENTS OF SODIUM FLUORIDE-TREATED DENTIN*

HOURS OF DECALCIFICATION	% WEIGHT LOST	% Ca	% P	% Mg.	% CO_2	% ELEMENTS RECOVERED
1	1.9	34.6	17.4	1.5	6.5	60.0
2	1.3	32.1	16.9	1.7	1.7	52.5
3	1.6	29.7	19.3	1.7	4.2	54.9
4	1.3	37.9	21.4	—	3.7	—
5	1.2	35.3	16.5	1.3	—	53.3
6	1.5	33.2	16.5	1.4	2.4	54.5
7	1.3	36.5	18.5	2.1	1.6	52.5
8	1.1	42.1	18.2	2.0	2.4	62.5
Mean and Standard Error	1.4 ± 0.08	35.2 ± 1.34	18.1 ± 0.59	1.7 ± 0.11	3.2 ± 0.67	55.7 ± 1.48

*Decalcifying agent: Morse solution.
(–) = No value obtained.

The rates of dissolution of the principal inorganic constituents of sodium fluoride-treated dentin are shown in Table V. The rate of decalcification of

sodium fluoride-treated dentin was very slow in comparison to that of water-treated or calcium fluoride-treated dentin, but the relative amounts of the inorganic elements released from sodium fluoride-treated dentin did not differ greatly from those released from water-treated control dentin or calcium fluoride-treated dentin. Of the total weight lost during decalcification, 55.7 ± 1.48 per cent was accounted for in the calcium, phosphorus, magnesium, and carbon dioxide recovered during the decalcification of sodium fluoride-treated dentin.

The ratios of the various elements released from sodium fluoride-treated dentin are shown in Table VI. Both the mean calcium:phosphorus ratio and the mean phosphorus:magnesium ratio were less than those of either water-treated control dentin or calcium fluoride-treated dentin. Considerable variation was found from hour to hour in the calcium:carbon dioxide ratio and in the phosphorus:magnesium ratio of the decalcifying solution of sodium fluoride-treated dentin.

TABLE VI
THE RATIOS OF VARIOUS ELEMENTS RELEASED FROM SODIUM FLUORIDE-TREATED DENTIN DURING DECALCIFICATION WITH MORSE SOLUTION

HOURS OF DECALCIFICATION	Ca:P	Ca:Mg.	Ca:CO_2	P:Mg.	Mg.:CO_2
1	1.99	23.2	5.3	2.6	0.22
2	1.89	18.3	18.3	9.6	1.00
3	1.54	17.3	7.0	4.5	0.40
4	1.77	—	10.1	5.7	—
5	2.14	26.7	—	—	—
6	2.02	23.4	14.0	6.9	0.60
7	1.98	17.5	22.0	11.1	1.25
8	2.32	20.6	17.9	7.7	0.86
Mean and Standard Error	1.95 ± 0.08	21.0 ± 1.34	13.5 ± 2.36	6.8 ± 1.14	0.72 ± 0.15

(–) = No value obtained.

DISCUSSION

In this study data were obtained which partially defines the relationship of the rate of dissolution of the principal inorganic constituents of control and fluoride-treated dentin to each other and to total weight lost during decalcification. These results indicate that while the amount of calcium, phosphorus, magnesium, and carbon dioxide recovered from the decalcifying solution does not account for the total weight lost from the tissues, the amount of the elements recovered is relatively constant for both untreated and fluoride-treated dentin and that the total weight lost is a fair indicator of tissue solubility. In fact, total weight loss is probably a better indicator of solubility than is the determination of the rate of release of any one of the elements or even better than the determination of a combination of any 2 elements. Even when determining the rate of release of a combination of 2 elements, discrepancies may arise during short periods of decalcification or between their rates of release during early or late decalcification or when the tissues are treated with different ions prior to decalcification. Therefore, determination of over-all weight changes during decalcification is perhaps as good a method for measuring the solubility

of dental tissues as is the determination of the rates of release of all of their principal inorganic constituents (calcium, phosphorus, magnesium, and carbon dioxide).

The data for the rate of removal of the elements during decalcification shown that the calcium:phosphorus ratio of water- and fluoride-treated dentin remains relatively constant. The dentin treated with calcium fluoride is a possible exception, for it may be that its calcium is more labile than that of untreated dentin. Since there is no increased loss of calcium during early decalcification of sodium fluoride-treated dentin, it is more likely that the labile calcium of calcium fluoride-treated dentin is absorbed or adsorbed onto it during treatment.

Several other differences are to be found in the ratios of the solubilized elements of water- and fluoride-treated dentin, the significance of which is not known. For instance, the mean phosphorus:magnesium ratio of sodium fluoride-treated dentin is different from that of the mean calcium fluoride-treated or control dentin, and the magnesium:carbon dioxide ratio of control dentin is different from that of either type of fluoride-treated dentin.

In spite of some differences in the rate of solubilization of the principal inorganic constituents of dentin in acid, it would appear that its treatment with fluorides affects the solubility of the molecular moiety, rather than affecting some particular element or part. The use of a calcium fluoride salt does influence the rate of dissolution of dentinal calcium during decalcification in comparison to that of phosphorus, but this action does not appear to be caused by the fluoride ion.

SUMMARY

The rate of dissolution by Morse solution of calcium, phosphorus, magnesium, and carbon dioxide of human dentin treated with distilled water, calcium fluoride, or sodium fluoride was determined at the end of 8 consecutive hourly intervals. The amount of the above elements recovered accounted for from 55 to 57 per cent of the total weight lost during decalcification and under the conditions of these experiments seems to be a fair indicator of solubility. The ratio between calcium and phosphorus remained relatively constant during decalcification of the different types of dentin, except that treatment of dentin with calcium fluoride resulted in an early increased release of calcium during decalcification. Some variations were found in the phosphorus:magnesium ratio and in the magnesium:carbon dioxide ratio of the decalcifying solutions of the different types of dentin.

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THE DISTRIBUTION OF FLUORIDE IN HUMAN ENAMEL

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IN A recent review, it was concluded that the mechanism of action of fluoride in reducing dental caries was its solubility-reducing effect on enamel.[1] Although this theory is supported by considerable laboratory evidence, it is contradicted by the observation of Volker[2] that mottled enamel is as soluble as normal enamel. Mottled teeth are caries resistant, and yet it was concluded that the fluoride content in the enamel of these teeth was insufficient to reduce the solubility. A reconciliation of Volker's finding with the above theory is only possible if the fluoride is unevenly distributed, that is, if it occurs in the outer enamel in concentrations capable of reducing the solubility. The purpose of the present study was to observe the distribution of fluoride in the enamel with the hope that a better understanding of its caries-reducing effect might be attained.

REVIEW OF LITERATURE

Only two investigators have been interested in fluoride distribution within the enamel. Schmid[3] found slightly more fluoride deposited in the outer than in the inner part of the enamel. Jenkins[4] found no difference between the fluoride content of the surface enamel and the rest of the enamel in deciduous teeth. However, in both unerupted and erupted permanent teeth he found at least five times more fluoride in the superficial fraction than in the remaining part of the enamel.[5] This last observation suggests that fluoride may accumulate in the outer enamel prior to eruption.

In addition to these findings there is a mass of evidence showing that fluoride is taken up by the external enamel. This is brought out by studies with radioactive fluoride,[6, 7, 8] with electron microscopy,[9, 10, 11] and by chemical determination of increased fluoride content of enamel after exposure to fluoride.[3, 12, 13, 14, 15, 16, 17] It is outside the scope of the present report to review all these studies and the additional evidence of uptake provided by clinical investigations. But the fact, demonstrated by radioactive fluoride, that the uptake is confined to the surface enamel[7, 8] together with all the other evidence of fluoride uptake, strongly suggests that high concentrations of fluoride may be found in the outermost part of the enamel.

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Although it may be assumed from the literature reviewed that greater amounts of fluoride occur in the outer than in the inner part of the enamel, detailed information about its distribution is lacking. In planning the present study, it was decided to determine the concentration of fluoride in several successive layers of enamel from both unerupted and erupted teeth of different ages. Deciduous teeth were included because it was considered desirable to check the finding that fluoride appears to be evenly distributed in these teeth. A group of teeth with mottled enamel was also studied in order to demonstrate the effect of high fluoride intake on the distribution of fluoride in the enamel.

TOOTH MATERIAL AND METHOD OF OBTAINING ENAMEL SAMPLES

Teeth collected from exodontists in Rochester, New York, included deciduous teeth, unerupted teeth, and erupted teeth which were divided according to age in the groups: under 20, 20 to 29, 30 to 49, and over 50 years of age. Because 1 ppm of fluoride had been added to the Rochester drinking water 1 year prior to this work, it was felt that data on unerupted teeth from a nonfluoride area would also be desirable. Therefore, unerupted teeth from Buffalo, New York, where the drinking water contains 0.1 ppm of fluoride, were included. Since a number of these teeth had incompletely formed roots, they were divided in 2 groups according to their development. In addition, mottled teeth of unknown age were obtained from a high fluoride area in Texas.

Since we were interested in the presence of lead, copper, and tin as well as fluoride, sufficiently large samples of enamel were collected to permit determination of all these elements. This was only possible by pooling samples from several teeth. Therefore, all teeth which were available were included with the result that the number of teeth and the distribution of tooth types in the different groups varied considerably. Table I gives a breakdown of the types and number of teeth included in the study. The ratio of carious to noncarious teeth also varied, with the greatest number of carious teeth occurring in the under-20-years-of-age group.

TABLE I
DISTRIBUTION OF TEETH IN GROUPS STUDIED

	UNERUPTED			ERUPTED				
	I*	II†	III‡	UNDER 20 YR.	20-29 YR.	30-49 YR.	OVER 50 YR.	MOTTLED§
Anteriors	0	4	2	28	45	78	54	0
Bicuspids	1	4	1	17	19	28	24	2
Molars	28	55	32	12	42	41	26	17
Total	29	63	35	57	106	147	104	19

*Not fully formed from Buffalo, N. Y.
†Fully formed from Buffalo, N. Y.
‡Fully formed from Rochester, N. Y.
§Age unknown.

Before use, the teeth were stored on aluminum trays in bottles containing cotton saturated with water and thymol. This procedure prevented the teeth from drying out and minimized bacterial growth without exposing the teeth

directly to the preservative. Dental restorations and carious material were removed with a bur, and the teeth were cleaned by gentle brushing with pumice and distilled water.

Samples of successive layers of enamel were obtained by grinding with a diamond stone. The ground enamel was collected in a porcelain crucible filter by means of suction, a procedure which reduced the loss of enamel during grinding to a minimum. The thinnest layers mechanically obtainable were ground from the outer surface in order to provide detailed information about the composition of this part of the enamel. Four to 9 successive layers were collected from each group of teeth. Therefore, in some groups only the outer part of the enamel was studied, while in others most of the enamel was included.

TABLE II
WEIGHTS IN MILLIGRAMS OF GROUND SAMPLES OF SUCCESSIVE LAYERS OF ENAMEL

LAYER NO.	UNERUPTED			ERUPTED				
	I*	II†	III‡	UNDER 20	20-29	30-39	OVER 50	MOTTLED
1	283	468	168	320	479	563	337	335
2	402	462	184	370	615	634	370	551
3	400	457	286	587	835	568	294	363
4	322	526	319	472	630	655	313	380
5			505	727	1,643		715	420
6			687		1,044		1,076	592
7			750		3,146§		1,391‖	440
8			841		2,789§			470
9			689		2,117§			442
Surface Score in the Different Groups of Teeth								
	65	130	69	79	160	210	150	40

*Not fully formed from Buffalo, N. Y.
†Fully formed from Buffalo, N. Y.
‡Fully formed from Rochester, N. Y.
§69 Teeth included; surface score 129.
‖75 Teeth included; surface score 108.

The weights of the ground samples are given in Table II. The first two layers generally weigh less than subsequent layers. The thickness of the outer layers was calculated to be thinner than 0.05 mm. in all groups except that of the mottled teeth. From these teeth, which were few in number, deeper layers had to be obtained to provide enough enamel for chemical analysis.

The data obtained on numerically equivalent layers in the different groups were not necessarily comparable because it was impossible to control exactly the thickness of enamel ground off in a single grinding. Since such a comparison was desired, it was necessary to consider the thickness of the enamel layers ground from different groups of teeth.

No exact method was available for measuring the thickness, but an approximate estimation was obtained by dividing the weight of each sample with the surface area of the teeth from which the sample was ground. The surface area was estimated as accurately as possible by giving each tooth a score ranging from 1.0 to 2.5, according to the size of the surface and adding the scores for each group. The total surface score for each group of teeth is

given at the bottom of Table II and the calculated score of thickness of the ground layers of enamel is tabulated in Table III. In evaluating the data, the fluoride concentrations of successive layers were plotted against the cumulative value of these scores which is equivalent to their depth.

TABLE III
THICKNESS SCORE OF LAYERS GROUND FROM THE DIFFERENT GROUPS OF TEETH

LAYER NO.	UNERUPTED			ERUPTED				
	I*	II†	III‡	UNDER 20	20-29	30-39	OVER 50	MOTTLED
1	4.3	3.6	2.4	3.3	3.0	2.7	2.2	8.4
2	6.2	3.6	2.6	3.9	3.8	3.0	2.5	11.6
3	6.2	3.5	4.1	6.1	5.2	2.7	2.0	9.0
4	4.9	4.1	4.6	4.9	3.9	3.1	2.1	9.5
5			7.2	7.6	10.2		4.8	10.4
6			9.8		6.5		3.8	14.8
7			10.7		25.0		7.1	11.0
8			12.0		22.2		12.9	11.7
9			8.9		17.0			11.0

*Not fully formed from Buffalo, N. Y.
†Fully formed from Buffalo, N. Y.
‡Fully formed from Rochester, N. Y.

METHOD OF FLUORINE DETERMINATION

For determination of fluorine, the enamel was ashed in nickel dishes at 575° C., and the fluoride separated by the distillation method of Willard and Winter.[18] The fluoride content of the distillate was determined by a titration method.[19]

TABLE IV
CONCENTRATION OF FLUORINE IN PARTS PER MILLION OF ASH IN SUCCESSIVE LAYERS OF ENAMEL

LAYER NO.	DECIDUOUS	UNERUPTED			ERUPTED				
		I*	II†	III‡	UNDER 20	20-29	30-39	OVER 50	MOTTLED§
1	448	331	528	847	571	1,205	1,070	1,247	1,859
2	126	101	232	391	269	531	553	667	754
3	43	57	150	201	112	324	368	404	470
4	56	33	96	172	90	233	249	315	372
5				88	48	180		176	328
6				64		77		147	261
7				40		67		97	233
8				26		77		58	228
9				32		105			228

*Not fully formed from Buffalo, N. Y.
†Fully formed from Buffalo, N. Y.
‡Fully formed from Rochester, N. Y.
§Age unknown.

RESULTS

The fluoride concentration found in the successive enamel layers of the different groups of teeth is given in Table IV. In all groups, a high concentration was found in the external enamel. The level of fluoride in the outermost layer was sometimes 10 times greater than the concentration of fluoride reported for whole enamel in the literature. The lowest fluoride concentration found in the external layer was 331 ppm and occurred in the

unerupted, not fully formed teeth from Buffalo. The surface enamel of fully formed unerupted teeth from Buffalo was higher in fluoride, 528 ppm, and still higher were the unerupted teeth from Rochester, with 847 ppm. The outer layer in nonfluorosed erupted teeth ranged from 448 ppm in deciduous teeth to 1,247 ppm in the over 50 years' group. Of the permanent teeth the under 20 years' group had the lowest figure, 571 ppm. As could be expected, the highest value, 1,859 ppm, was found in the mottled teeth.

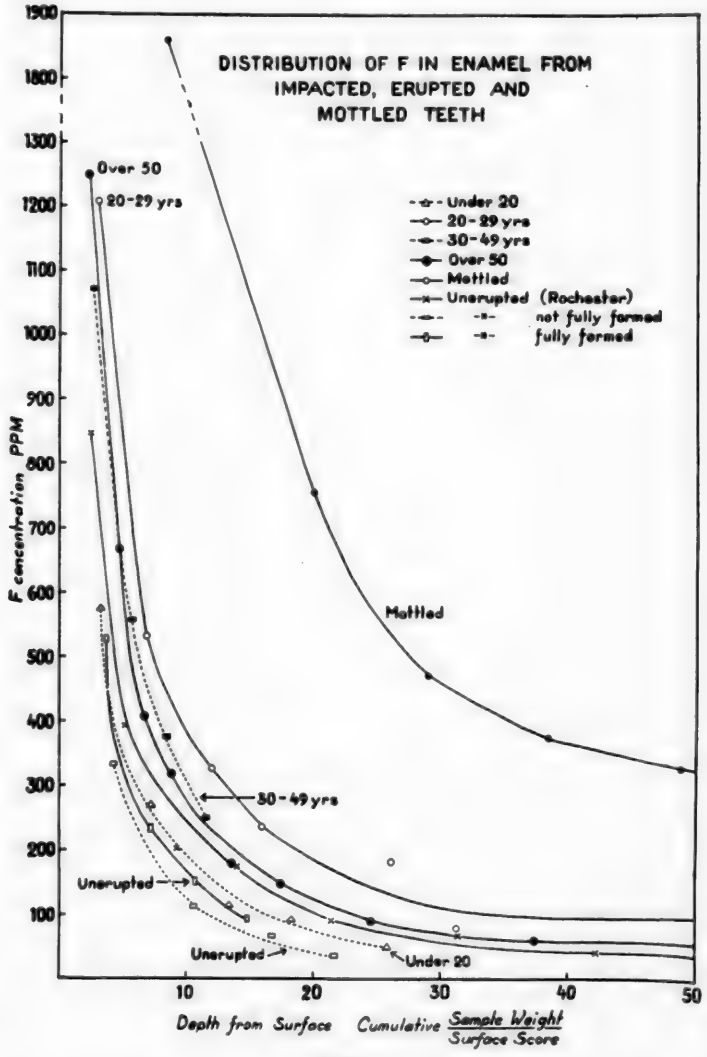

Fig. 1.

There is a steady decrease in the fluoride concentration from the surface inward in all groups of teeth. Complete data are not available on all the teeth, but the findings bear out the fact that the fluoride concentration decreases

with increasing enamel depth, until a level is reached where the concentration remains fairly constant. In the unerupted, fully formed Rochester teeth, the concentration in this inner portion appears to be somewhere between 30 and 60 ppm. In mottled enamel, the corresponding concentration was 240 ppm or approximately 4 times greater than that of normal enamel.

An increase can be noted in the fluoride concentration of the fourth layer in the deciduous teeth and in the ninth layer in the 20 to 29 years' group. It is not known whether this represents a real increase in fluoride in the enamel toward the dentinoenamel junction, or whether the last samples became contaminated with dentin, which is known to contain more fluoride than enamel.

The fluoride values found in the different groups indicate a general increase in the concentration with age both in the unerupted and erupted teeth. However, as has already been pointed out, a comparison of these data is not necessarily valid because of the different thicknesses of the various enamel layers. For this reason, the fluoride concentration of successive layers was plotted against the derived cumulative thickness or distance from the surface inward of these layers (Table III). The resulting curves, which make a comparison possible, are shown in Fig. 1. These curves represent the distribution of fluoride from the surface inward in the enamel of the different groups of teeth as accurately as our methods permit. There appears to be a conspicuous build-up of fluoride in the outer enamel of unerupted teeth as shown by the difference in the curves of incompletely formed and fully formed teeth. The pattern of distribution is surprisingly similar in all the teeth from Rochester. There appears to be some increase in fluoride concentration with age in the outer layer of the enamel, but in the bulk of the enamel, the level is about the same in all age groups, suggesting no change in this part of the enamel. The 20 to 29 years' group shows somewhat higher concentrations than the other groups. It is possible that some teeth in this group came from persons who at one time lived in high fluoride areas; the history of the individual teeth was unknown.

The curve of the data from the mottled teeth is outstandingly different in that it shows a much higher concentration of fluoride at any distance from the surface than was found for normal teeth. The curve is somewhat flatter, indicating that the very high concentrations of fluoride which are present only in the outermost layer of normal teeth extend further into the enamel of mottled teeth.

Differences in fluoride distribution among the various groups of teeth are more clearly shown in Fig. 2, where the logarithm of the fluoride concentrations is plotted against the derived distance from the surface. It is possible to estimate the fluoride concentration in the outer molecular layers of the enamel by extrapolating the curves of Fig. 2 to the points where they cross the ordinate. The extrapolated concentrations (as ppm F) approximate a fluoride concentration of 1 per cent for the teeth of each group. If fluorapatite is assumed to contain about 3.6 per cent fluoride, it appears that the enamel surface is approximately one-fourth saturated with respect to fluoride, regardless of age.

Rathje,[20] in studies on the solubility of hydroxylapatite-fluorapatite mixtures, has shown that the least soluble mixture is that one containing 20 per cent of fluorapatite. The fluoride concentration of this mixture approximates 0.7 per cent which agrees well with the concentration estimated from our data for the outermost enamel layer.

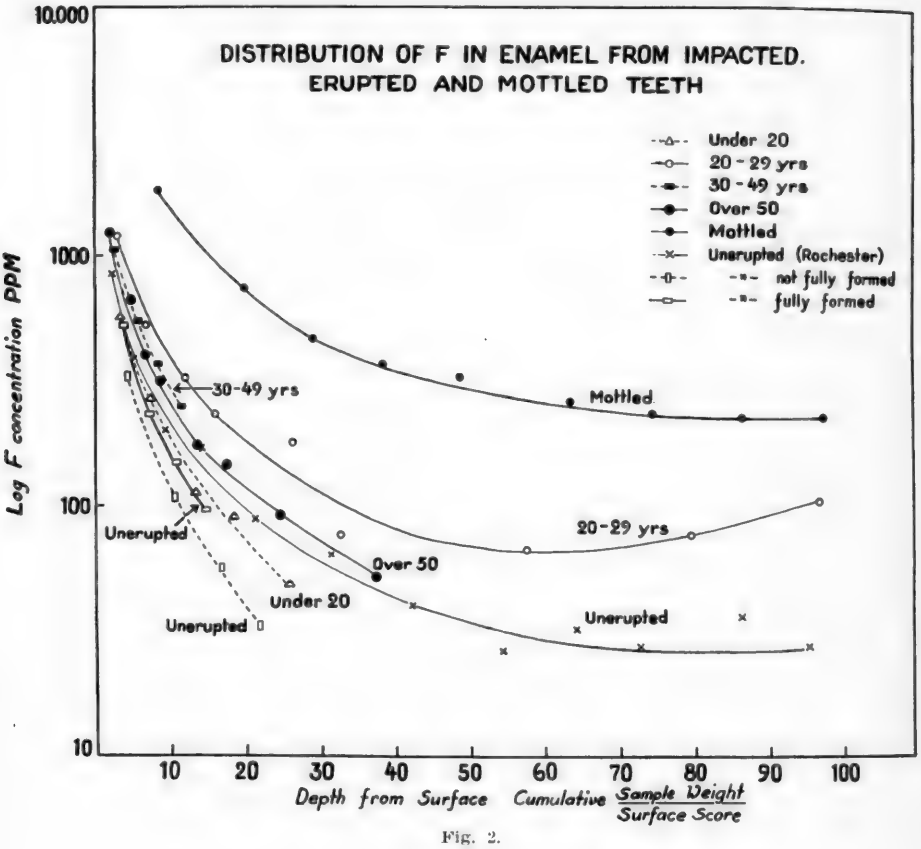

Fig. 2.

DISCUSSION

Our findings are in agreement with Jenkins' observation that the concentration of fluoride is greater in the surface layer than in the remainder of the enamel, in both erupted and unerupted teeth. Contrary to Jenkins, we also found an accumulation of fluoride in the outer enamel of deciduous teeth. From our data, it appears that fluoride deposition in the enamel may be considered to take place in 3 stages:

1. Small amounts are deposited throughout the enamel matrix during the period of calcification.

2. After calcification is completed, the bulk of the enamel loses contact with the tissue fluids and, as a result, the fluoride acquisition from tissue fluids is

limited to the external surface. This part of the enamel continues to pick up fluoride until eruption, and it is likely that the outer crystal layers of the enamel may reach a high degree of fluoride saturation in the pre-eruptive period, provided the fluoride level is high enough and the period between tooth formation and eruption is sufficiently prolonged.

3. Our data also suggest that fluoride uptake may continue after eruption, but this uptake is also confined to the external part of the enamel. There is no evidence that appreciable amounts of fluoride will penetrate more than 0.1 mm. into intact enamel in the pre-eruptive or posteruptive period.

In all groups of teeth, the fluoride concentration in the outer crystal layers of the enamel appears to be approaching the 1 per cent level. It is likely that this concentration represents the saturation point under physiological conditions and that it is reached relatively early in the life span of a tooth by the surfaces which are in intimate contact with tissue fluids. Continued increase in fluoride content of the enamel apparently takes place, not by further increase in the concentration of the outer crystal layer, but by a movement of fluoride ions in depth from highly saturated to less saturated crystallites. In this way, a slow but gradual thickening of the saturated outer enamel layer may occur with age.

It is likely that the caries-inhibiting effect of fluoride is associated with the high concentrations in the surface and that the lower levels of fluorine in the subsurface enamel provide little protection. This concept is supported by studies concerned with the effect of fluoride on enamel solubility and on bacterial growth.

As already mentioned, Volker found no difference in the solubility of normal and mottled powdered human enamel and concluded that the presence of fluoride in large amounts may decrease the solubility of enamel, but that small amounts show no demonstrable reduction.[2] These small amounts in the subsurface layers of mottled enamel, according to our findings, amounted to approximately 300 ppm of fluoride. Only in the surface enamel of both mottled and normal enamel is fluoride found in large amounts, and, incidentally, surface enamel is considerably less soluble in acid than subsurface enamel.[21]

With regard to the antibacterial action of fluoride, it has been found that concentrations in excess of 250 ppm are needed to affect bacterial growth.[22] Therefore, whether the mechanism of action of fluoride in reducing caries is one of reducing acid solubility or affecting bacterial growth, it is evident that the high concentrations present in the surface are more likely to be effective than the much lower concentrations in the subsurface enamel.

It is of interest that attempts to correlate the fluoride content of enamel with susceptibility to dental caries have met with varying degrees of success. Caries-free teeth have been reported, by some workers,[22, 24, 25] to have more fluoride in the enamel than carious teeth, but in the most comprehensive study made to date,[26] no relation was found. It is likely that a more consistent

correlation with dental caries may be established if the fluoride concentration in only the outer part of the enamel, rather than in the entire enamel, is considered.

Because of the high saturation of fluoride in the external enamel, it is interesting to speculate on the mechanism of action of topical application of fluoride in reducing dental caries. A radioactive fluoride study has shown that fluoride is primarily picked up in pathological areas of the enamel.[7] Evidently, these areas have either been depleted of fluoride because of the acid condition associated with the carious attack, or part of the outer enamel has been destroyed, resulting in exposure of subsurface enamel which is low in fluoride. In either case, a selective uptake by these areas is understandable. The possibility also exists, of course, that particularly in young teeth from low-fluoride areas, the surface enamel has not reached the saturation point and, therefore, fluoride is picked up by the entire enamel surface.

The difference in the fluoride content of mottled and normal enamel warrants some discussion. The finding that high concentrations of fluoride extend further into the subsurface enamel in mottled teeth is probably related to at least 2 factors. One of these, obviously, is the greater amount of fluoride which is present in water, food, and tissue fluids in areas of endemic fluorosis. Second, defects were conspicuous in the outer enamel of the mottled teeth. This enamel was chalky and soft to grind, indicating a loosely bound structure. It would seem that penetration of fluoride is facilitated in this type of enamel and that, therefore, the defective enamel contributed to the deeper impregnation of fluoride in these teeth.

SUMMARY

1. The distribution of fluoride has been studied in successive layers from the surface inward in the enamel of deciduous teeth, and of permanent unerupted and erupted teeth of different age, and of teeth with mottled enamel.

2. In all these teeth a high concentration of fluoride was found in the outermost layer of the enamel and a low level was found in the bulk of the enamel.

3. The findings suggest that small amounts of fluoride are laid down in the enamel during formation, and that only the outermost enamel picks up fluoride once calcification has been completed.

4. Appreciable amounts of fluoride accumulate in the outer enamel of unerupted teeth. There also appears to be a posteruptive pickup. The outermost crystal layers of enamel may reach fluoride concentrations as great as 1 per cent.

5. Deciduous teeth contain less fluoride in the outer enamel than permanent teeth.

6. Mottled enamel contains higher concentrations of fluoride than that of normal teeth at any distance from the enamel surface.

7. The caries-reducing effect of fluoride is believed to be due to the high concentration of fluoride in the surface enamel. Little protection is believed to be afforded by the low concentrations which were found in the bulk of the enamel.

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THE DISTRIBUTION OF LEAD IN HUMAN ENAMEL

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THE studies which have been made on the occurrence of lead in normal teeth suggest that this element is consistently present in small amounts.[1-7] The normal level for whole ashed teeth has been reported to be in the range of 20 to 70 ppm.[3, 4] Dentin appears to contain approximately 3 times more lead than enamel.[3] There have been no studies on the presence of lead in unerupted teeth or on uptake of lead by teeth. Therefore, it is not known to what extent lead found in teeth has been incorporated during tooth formation or deposited after eruption. The fact that concentrations of lead above normal have been found in the teeth of persons suffering from lead poisoning[1, 3, 7, 8, 9, 10] demonstrates a posteruptive uptake. Lead determinations in normal teeth of different ages also suggest a posteruptive gain up to the age of about 20, when a fairly constant concentration is reached.[3, 4] A similar condition apparently exists with regard to lead in bone, in that there appears to be no definite increase with age after a state of equilibrium has been reached between uptake and loss.[11]

The significance of the presence of lead in teeth has not been established. It has been suggested that lead may be harmful because caries was found to develop in a cat suffering from lead poisoning.[1] More significant is the finding that the number of DMF teeth in workers in lead plants was about the same as that in other industrial workers.[12] Topical application of lead fluoride has been ineffective in reducing dental caries. Two studies gave negative results[13, 14] and, in a third study, the reduction was no greater than has been observed with sodium fluoride.[15]

In spite of these negative findings, it is possible that the presence of lead may render the teeth more resistant to acid decalcification. This is suggested by the observation that powdered enamel exposed to lead salts has a decreased solubility.[16-20] However, the solubility-reducing effect of lead needs further investigation because of the contradictory findings which have been reported on intact enamel. Exposure of the enamel surface to lead has been found to produce either good[21] or fair[22] reduction, or, in the case of our own experiments, negative results.[23]

Although the evidence of the importance of lead with regard to dental caries is negative, the studies in this field have by no means been exhaustive. The fact that lead normally tends to accumulate in bone and teeth always raises

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the possibility that it may exert an effect in the mouth under certain conditions. For this reason, it was felt that a study of the concentration and distribution of lead in enamel from teeth of different ages would be of interest.

METHOD

Following a previously described procedure[24] ground samples of successive layers of enamel were pooled from partly and fully formed unerupted teeth, and from erupted teeth representing persons of the age groups under 20, 20 to 29, 30 to 49, and over 50 years of age. A sufficiently large number of teeth was included to provide enamel for determinations of fluoride, copper, and tin as well as lead. These additional results are reported in separate papers with a description of the tooth material included in the paper on fluoride.[24]

Lead was determined as follows: For samples containing high concentrations, 40 mg. of enamel was dissolved in dilute hydrochloric acid to give 4 mg. per 0.1 ml. Spectrographic carbons were impregnated with 0.1 ml. amounts of this solution or of the solution suitably diluted. For low concentrations, 10 mg. of the tooth material was added dry to each carbon. Lead was evaluated by the spectrum line 2833.07 A. The internal standard was bismuth; 0.25 Gm. was used, and the line read was 2897.98 A. Excitation of the sample was by the conventional DC carbon arc. Analyses were done in quadruplicate.

RESULTS

Table I shows the concentration of lead in successive layers of enamel from the different groups of impacted and erupted teeth. The amount of lead in the outer enamel of mottled teeth of unknown age from Texas is also given.

TABLE I
CONCENTRATION OF LEAD IN PARTS PER MILLION IN SUCCESSIVE LAYERS OF ENAMEL

	UNERUPTED		ERUPTED				
LAYER NO.	I*	II†	UNDER 20	20-29	30-49	OVER 50	MOTTLED
1	180	210	350	360	520	550	260
2	100	130	220	260	430	460	70
3	55	67	180	105	280	420	52
4	40	60	74	85	200	310	52
5		55	35	65		156	
6		47		54		152	
7		44		47		114	
8		33		42		95	
9		29		34			

*I = Incompletely formed.
†II = Fully formed.

The unerupted as well as the erupted teeth showed fairly high concentrations of lead in the external layer of the enamel and rapidly decreasing values in successive layers of subsurface enamel. The amount of lead in the surface enamel was greater in erupted than in unerupted teeth. In the latter the lowest concentration, 180 ppm, was found in the incompletely formed teeth, compared to 210 ppm in the fully formed ones. Among the erupted teeth the ones under 20 years with 350 ppm and the 20 to 29 years' group with 360 ppm

were similar, while the two older groups, the 30 to 49 with 520 ppm and the over 50 with 550 ppm had much higher concentrations. The mottled teeth from Texas showed lower lead values than any of the erupted teeth from Rochester, demonstrating the fact that teeth from different geographical areas

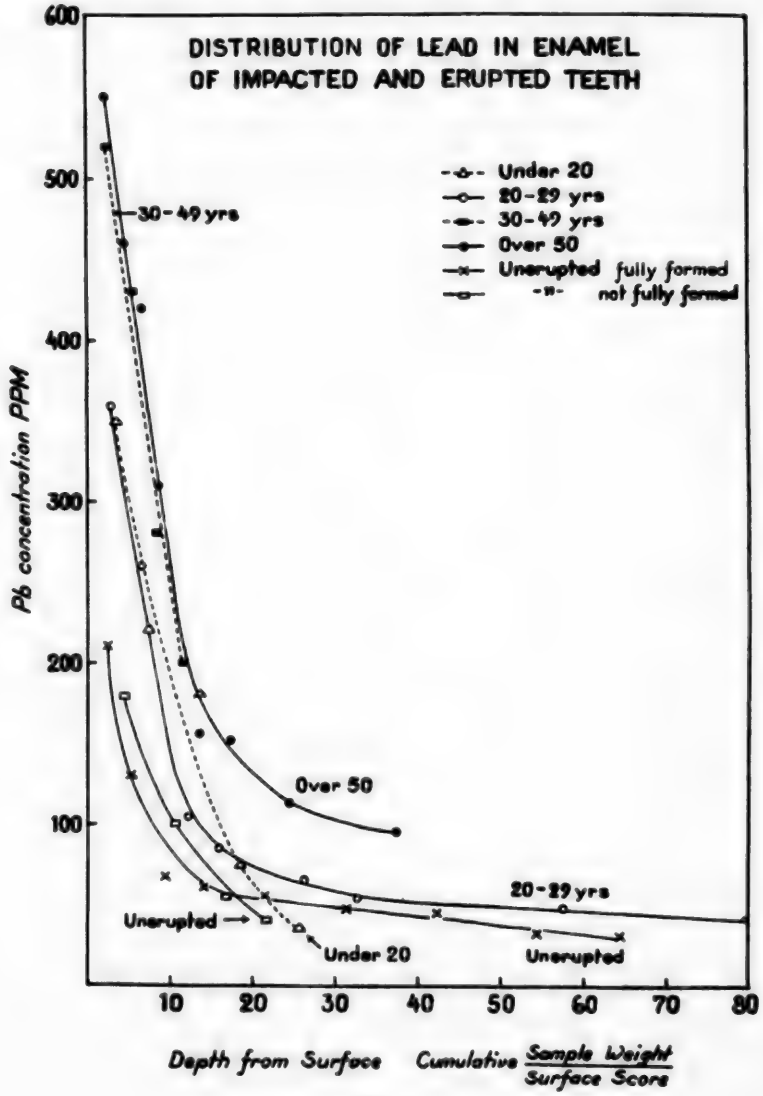

Fig. 1.

may vary in the content of lead. It is possible, also, that the great amount of fluorine in the mottled enamel may interfere with lead uptake. In all groups, the concentration of lead in the external enamel was many times greater than that reported in the literature for whole teeth. However, the concentration of

lead decreased rapidly from the surface inward, and the bulk of the subsurface enamel contained concentrations of one-fifth or less than that of the external layer.

The possibility was considered that the great amount of lead which was found in the outer layer of the enamel may, in part, have originated from dental restorations and that we were dealing with a contaminant and not a constituent of the enamel structure. To check this possibility, ground enamel samples of 5 successive layers from intact teeth of persons over 50 years of

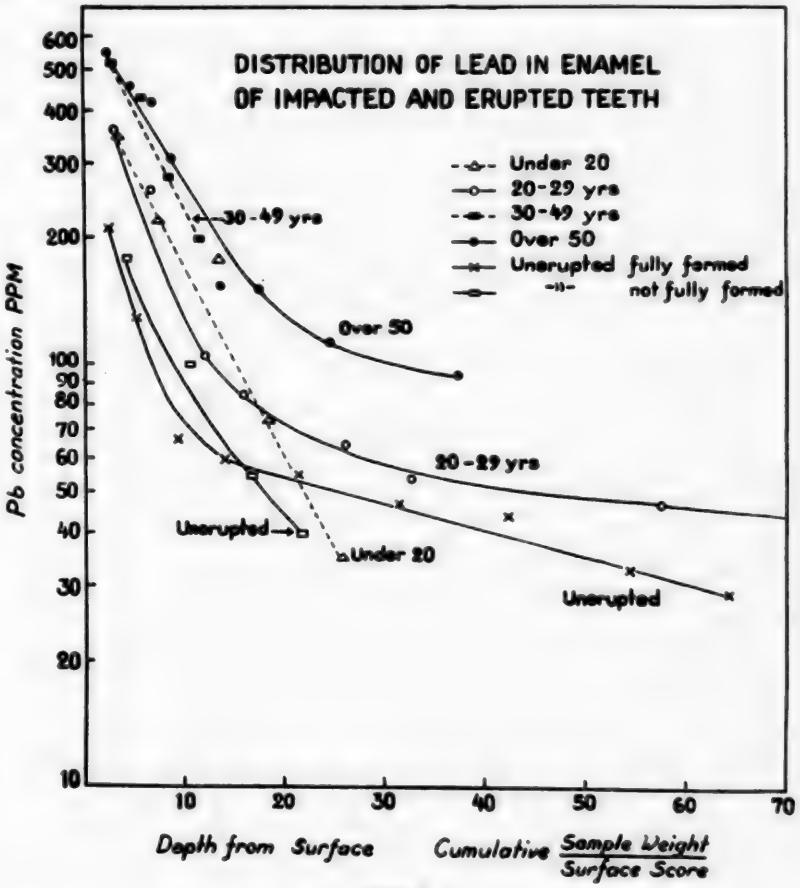

Fig. 2.

age were subjected to lead determination. The findings were 500, 340, 210, 90, and 70 ppm of lead in the respective layers, taken in order from the surface and inward. As a result of these observations, it was concluded that lead may be normally present in intact enamel in the concentrations which we found.

In order to compare the distribution of lead in the different groups of teeth, the lead concentrations of the successive enamel layers were plotted against the distance from the surface, using the same procedures as previously described

for fluoride.[24] In Fig. 1, it will be seen that the pattern of distribution is similar in all groups. There is a high concentration of lead at the enamel surface, and rapidly decreasing concentrations in subsurface enamel toward a fairly stable level, which is characteristic for the bulk of this enamel.

From the curves of the impacted teeth, it is evident that appreciable amounts of lead are picked up by the enamel before eruption, but the high concentrations found in the older erupted teeth suggest that there is also a post-eruptive lead acquisition.

In Fig. 2, the logarithms of the lead concentrations in the successive enamel layers are plotted against the depth from the surface. From this chart, the concentration of lead in the outer molecular layer of the enamel may be estimated by extrapolating the different curves to the ordinate. In most groups of teeth, this outer superficial concentration appears to be in the order of 700 ppm.

DISCUSSION

The present study has confirmed the observation of other workers that lead is a consistent constituent of teeth. In addition, it shows that the concentration of lead may be up to 20 times greater in the outer part of the enamel than that found in the entire enamel in previous studies.

The pattern of distribution of lead in the enamel is conspicuously like that of fluorine, both in unerupted and erupted teeth. Therefore, certain aspects of the uptake of these 2 elements by the enamel must be similar. It was postulated in the case of fluorine that the fully calcified enamel is practically impermeable to the fluorine ion.[24] The same is evidently true of lead. According to this view, the lead found in the bulk of the enamel must have been laid down in the enamel matrix before calcification was completed. That lead is readily taken up during this period is the more likely because of the observation on bone that structures which undergo calcification pick up lead at a greater rate than fully formed structures.[25, 26] Once calcification has been completed, lead acquisition occurs primarily from the outer surface, and, because of an exceedingly slow penetration into the enamel, high concentrations accumulate in the outer enamel.

Posteruptive gain in lead is also suggested by the high concentration found in the outer enamel of the older teeth. Such a gain is in accord with observations of increased lead content in teeth from persons suffering from lead poisoning and in teeth from animals on a high lead diet.[3] In considering the post-eruptive uptake of lead, it should be borne in mind that substantial amounts of lead may have been deposited in developing teeth some decades ago because of the greater hazards of lead exposure then than nowadays. Therefore, the increase after eruption may not be as great as indicated by our data.

Previous studies suggest that an equilibrium between uptake and loss of lead in teeth may be reached early in adult life. The complexity of this problem of uptake and equilibrium is brought out by our procedure of studying the surface layers of the enamel, rather than entire teeth as was done in the past. The high concentrations found in the outermost layer of teeth from the older age groups may very well represent a saturation or equilibrium level under

conditions existing in the mouth, but the much lower concentrations in subsurface enamel certainly show that the bulk of the enamel is far from being saturated with lead. Therefore, since small amounts of lead are present in the average diet,[27] it is likely that the lead content in the enamel will increase slowly with age. This, as a matter of fact, is what our findings indicate will happen.

The presence of lead in the enamel must depend not only on the uptake and rate of penetration, but also on loss. No studies have been done on the mobility of lead in the enamel, but studies on bone indicate that lead may be easily withdrawn from calcified structures. For instance, citrate, acid, or alkaline agents will deplete bone of lead, as shown by increased urinary output,[28] and, under certain conditions, lead may actually be mobilized from bone more easily than calcium.[29] The oral environment must generally be unfavorable for mobilization of lead, for, otherwise, the high concentrations found in the surface of intact enamel would be inconceivable. But, in view of the findings cited above, it is likely that lead may be lost from surface areas where acid conditions prevail, and it seems doubtful whether the presence of lead will contribute materially toward making the enamel more resistant to the action of acid. Some evidence that lead will leach from enamel faster than phosphate at pH 4 has been obtained in separate tests in our laboratory. This was concluded from the finding that the lead-phosphorus ratio of the outer layer of enamel, etched off in an acetate buffer, was higher than would be expected. Evidently, at least at this low pH, lead is loosely bound in the enamel and will go into solution faster than some of the other constituents of the enamel. However, the effect of lead on solubility needs further clarification. For instance, our own solubility tests showed no decrease in solubility after exposure of intact enamel surfaces to lead nitrate but a marked decrease when etched enamel surfaces were similarly treated.[30] Also as already mentioned in the literature review, several investigators have shown that lead salts will reduce the solubility of powdered enamel. It is possible that both the amount of lead present and the type of bond with which it is attached to the enamel may be important factors with regard to acid solubility.

With regard to the nature of the deposition of lead in the enamel, nothing is known, but studies on bone have shown that it will exchange with calcium.[1] More recent work indicates that lead may first absorb on the surface of the apatite crystallites and that it may eventually assume a position within the crystalline structure.[31]

SUMMARY

1. The distribution of lead was determined in pooled ground samples of successive layers of enamel from unerupted and erupted teeth of different ages. A group of mottled teeth was also included.

2. Lead was found to be present in the enamel of both unerupted and erupted teeth.

3. There was an increase in the lead concentration of the surface enamel in fully formed unerupted teeth, compared to that of incompletely formed ones.

The amount of lead in the outer enamel of erupted teeth was greater than that of unerupted teeth and showed an increase with age. The greatest concentration in the outermost enamel, 550 ppm, was found in the over 50 years' group.

4. In all groups of teeth, the greatest amount of lead was found in the outermost enamel layer, and decreasing amounts were found in the subsurface layers until a fairly constant level was reached which represented the lead concentration in the bulk of the enamel. This concentration was about 30 ppm in teeth from young persons and about 90 ppm in teeth from persons over 50 years of age.

5. The lead content of the enamel of mottled teeth was less than that of normal erupted teeth, showing either that the amount of lead in the enamel may vary in different geographical regions or that presence of fluorine in large amounts may interfere with lead uptake.

6. Since lead occurs in relatively high concentrations in the outer enamel, its presence may be important, but, as yet, its significance has not been established.

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AMMONIA PRODUCTION IN SALIVA II.

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PREVIOUS work[1, 2] has shown that there is an ammonia-producing mechanism in saliva which is inhibited by glucose. This glucose inhibition was still exhibited when solutions buffered at pH 7.0 were used, showing that the inhibition was not altogether due to a lowering of the pH.

A urease which produces large amounts of ammonia when urea is added is also present in saliva. If glucose is added to these urea-saliva mixtures, the ammonia production is greater than when urea alone is used.

When the pH is maintained at pH 7.0, the stimulating effect of glucose on ammonia production in the presence of urea is no longer observed. This would indicate that pH is the decisive factor and that glucose acts to maintain the pH near the optimum for ammonia production and does not exert any stimulating effect by itself.

EXPERIMENTAL

Wide variations in the final concentration of glucose (without added urea) from 0.1 to 10 per cent produced comparable drops in pH on incubation with saliva. The concentration of glucose does not seem to be related to the drop in pH at these levels.

Wide variations in the final concentration of urea (without added glucose) from 0.1 to 5 per cent produced comparable amounts of ammonia, and the pH rose from 7.5 to 9.0 in all tubes. However, 10 per cent urea inhibited ammonia production.

The effect of varying the ratio of glucose to urea on ammonia production was investigated and the results are shown in Table I.

TABLE I

THE EFFECT OF VARIATIONS IN THE RATIO OF GLUCOSE TO UREA ON AMMONIA PRODUCTION IN SALIVA

TUBE NO.	CONCENTRATION		RATIO GLUCOSE/UREA	FINAL pH	NH_3 (MG. PER CENT)
	GLUCOSE (PER CENT)	UREA (PER CENT)			
1	4.0	0.2	1:0.05	5.6	235
2	2.0	0.2	1:0.1	5.4	270
3	5.0	1.0	1:0.2	8.6	277
4	1.0	1.0	1:1	8.8	283
5	1.0	3.0	1:3	8.3	245
6	1.0	5.0	1:5	8.7	70
7	2.0	-	-	4.0	none
8	-	1.0	-	9.0	190

These results show that when the urea concentration falls below $\frac{1}{10}$ of the glucose it is not sufficient to prevent a drop in pH even though the ammonia production stays at a fairly high level. Excessive amounts of urea in the presence of glucose inhibited ammonia production.

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The amounts of glucose and urea used in most of the earlier experiments (2 per cent glucose and 1.5 per cent urea) resulted fortuitously in a ratio of glucose to urea which gave the optimum stimulation of ammonia production by glucose.

SUMMARY

In an attempt to shed further light on the ammonia-producing mechanism in saliva, experiments were performed in which the concentrations, when used separately, and the ratios, when used in conjunction, of urea and glucose added to the saliva were varied. Only small amounts (0.1 per cent) of glucose (no added urea) were needed to obtain large drops in pH. Concentrations of urea (no added glucose) below 0.3 per cent did not cause a rise in pH on incubation. High concentrations of urea (10 per cent) inhibited ammonia production.

A ratio of glucose to urea of about 1:1 exhibits optimum stimulation of ammonia production. If the urea is lowered below 0.2 per cent (added glucose), the drop in pH is not prevented. If the urea is raised above 3 per cent, inhibition in ammonia production is observed.

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THE CULTIVATION OF TOOTH GERMS IN AN EMBRYO EXTRACT-FREE MEDIUM

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EXPERIENCES in the in vitro cultivation of tooth germs have established that growth and development are most rapid in the first days of cultivation.[1-5] In these studies, the media contained embryo extract which is known to possess growth stimulating substances.[6] Chick embryo extract, for rat and mouse molar tooth germ explants, seemed to provide nutritional requirements for 4 to 8 days, after which degeneration occurred.[1-3] Dilute homologous embryonic extract provided rat molar tooth germ explants with more desirable medium in which growth and development continued beyond 8 days at a retarded rate.[3] In the latter studies, survival ensued for the arbitrarily chosen period. These findings suggest that at the time of planting there is a residual nutrient medium in the organ which, until consumed, is capable of sustaining growth and development. It was also deemed important to establish the efficacy of embryo extract for tooth germ cultivation. This was achieved by comparing the results of this investigation with similar periods of cultivation in media containing embryo extract.[4] The following study was executed as a guide to evaluate the survival, growth, and development potential of the residual nutritional media in the explants. It was found that tooth germs, cultivated in embryo extract-free media, had an optimum survival period of 4 days in vitro and degenerated after 8 days of cultivation.

MATERIALS AND METHODS

Twenty-one tooth germs dissected from 19-day embryos[7, 8] of Rockland albino rats were cultivated on the clot in Carrel flasks. The 1 c.c. coagulum consisted of 3 parts of chicken plasma and 1 part of Tyrode's solution. The 1 c.c. supernatant fluid was Tyrode's solution. Both the coagulum and supernatant fluid contained .005 phenol red. The medium was adjusted to a pH of 7.6 at the time of planting. All cultures were washed with Tyrode's solution for 2 hours and fresh Tyrode's solution added every 4 days. The pH was adjusted to 7.6 at this time. Photographs of the explants were taken every 4 days. Observations made during cultivation were concerned with survival, growth, and development of the tooth germ. Changes in morphology, opacity, and the outgrowth characteristics were also recorded. Cultivation was terminated at 4-day intervals up to 16 days for histologic examination which supplied additional information on survival and development. The histologic

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technic was that used in previous studies.[9] This investigation parallels a study already reported in which embryo extract was included in the coagulum and supernatant fluid.[4] The findings were also compared with the normal in vivo development of the rat molar tooth germ.[7]

RESULTS

When observed in the flask most of the explants appeared to survive from 4 to 8 days. After this period, 10 of the 21 explants completely degenerated in the flask so that only 11 cultures provided tissue for histologic examination.

Most of the explants remained opaque for the duration of cultivation so that it was impossible to observe development. Slight growth was observed in the first days of cultivation, although it was markedly less than that which occurred in a similar group containing embryo extract.[4] One explant showed growth after 12 days of cultivation, but had completely degenerated at 16 days. In general, shrinkage of the explant occurred after 8 days of cultivation.

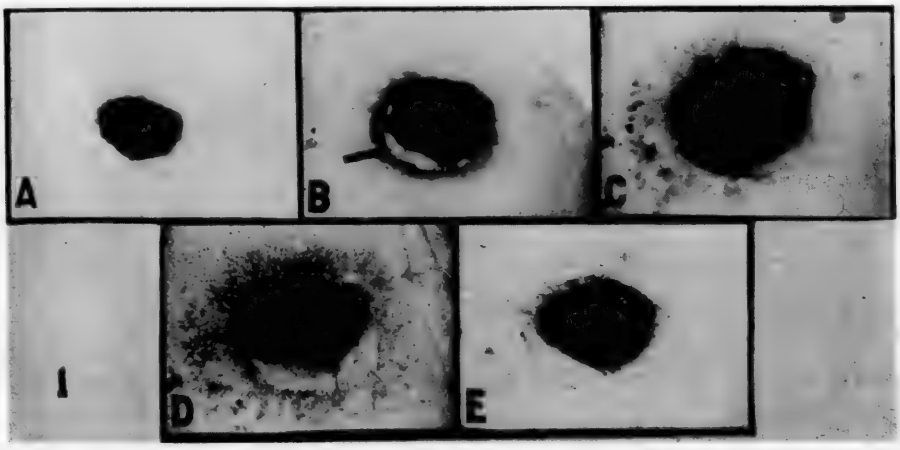
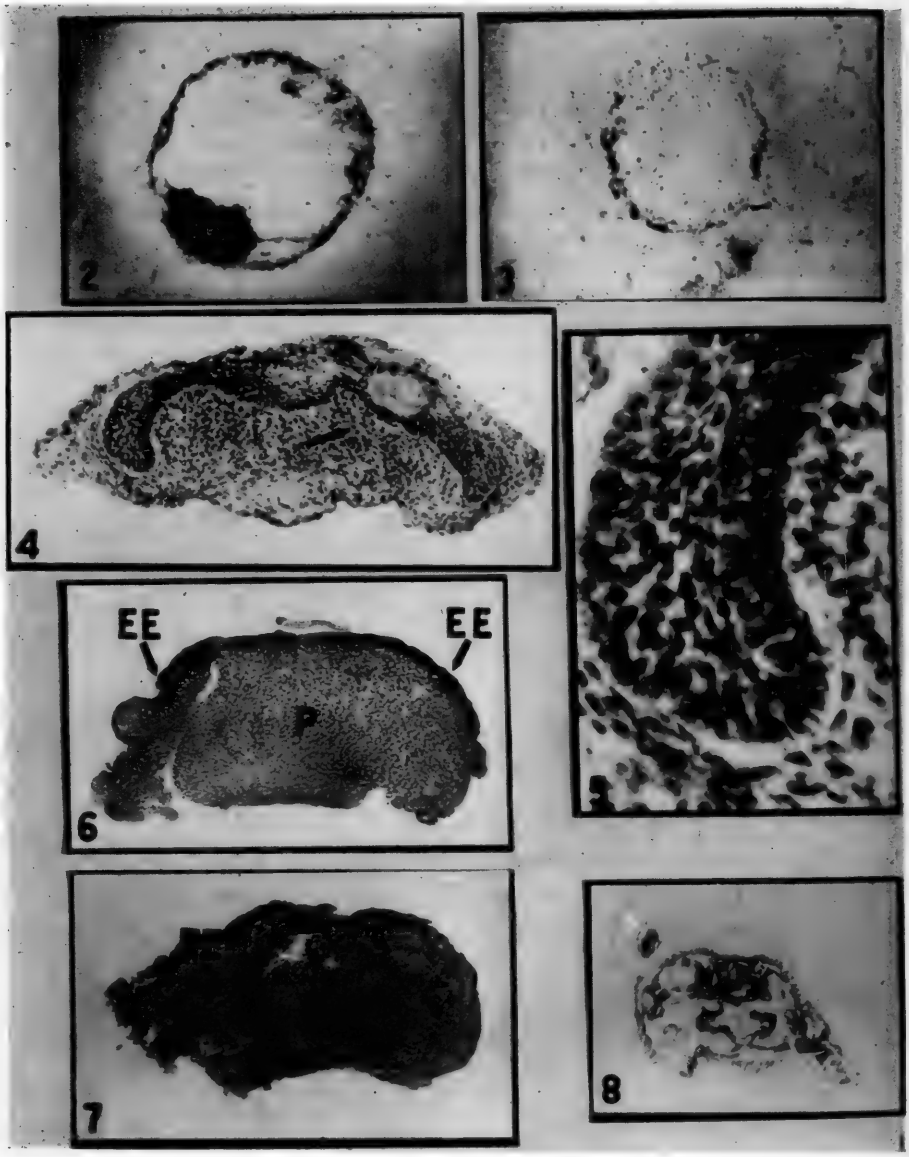


Fig. 1.—Nineteen-day rat embryo molar tooth germ cultivated in an embryo extract-free medium photographed in Carrel flask. *A*, 1 day in flask; *B*, 5 days in flask, arrow points to degenerated outgrowth; *C*, 9 days in flask, note degenerated outgrowth; *D*, 12 days in flask, outgrowth consists of fibroblasts and monocyte-like cells; *E*, 16 days in flask, note degenerated tooth germ and outgrowth. (Orig. mag. ×35.)

There was no common pattern of outgrowth. Three explants which exhibited no outgrowth after 4 days were designated as necrotic either upon or immediately after planting. In 18 instances, the presence of outgrowth in 4 days indicated survival of the peripheral cells of the explant. For 8 days outgrowth increased at a retarded rate using, as normal, the in vitro rate observed with media containing embryo extract.[4] At this time, the innermost cells of the outgrowth degenerated (Fig. 1). The peripheral cells continued to divide at a greatly retarded rate. The coagulum in the area of degenerated cells liquefied and monocytes appeared in the area. The liquefaction of the clot and lysis of the outgrowth proceeded both toward the periphery and centrally, so that the process finally involved the tooth germ. Ultimately only a



Figs. 2-8.—*(For legends see opposite page.)*

ring of monocyte-like cells and young fibroblasts remained (Fig. 2). The process of liquefaction completely destroyed the tissue in 10 explants. Of these, only 1 case showed survival of the monocyte-like cells at 16 days of cultivation (Fig. 3).

The 11 explants which provided tissue for histologic study were examined grossly at the time the specimens were taken and surprisingly retained their egg-shaped morphology. All cultures were sectioned, including those which obviously had degenerated in the flasks. The latter showed only plasma after histologic examination.

Two explants were taken for histologic examination at the end of 4 days of cultivation. In 1 specimen the enamel organ dedifferentiated into stratified squamous epithelium and the dental papilla showed degenerative changes. The few identifiable fibroblasts showed karyorrhexis or pyknotic nuclei. Fig. 4 is a photomicrograph of a section of the other specimen which exhibited greater survival and development. In this case the enamel epithelium retained its normal pattern. Cell division continued in the proliferative zone (Fig. 5). Two epithelial pearls developed in the central cell area, a phenomenon which also occurred in tooth germs cultivated in media containing embryo extract.[3, 4] The papilla survived with the exception of a small centrally located area of degeneration. Beginning differentiation of odontoblasts may be observed in one area of the papilla. As measured by in vivo development, the explant demonstrated an advance in development of 1 day.

Of the 10 tooth germs cultivated for 8 days, only 3 showed tissue after sectioning. In general, the epithelium again exhibited greater survival than the papilla. Fig. 6 is a section typifying the average result. The degenerated enamel epithelium, which resembled stratified squamous epithelium with hornification, indicates dedifferentiation of the enamel organ. The papilla presented a mass of degenerated fibroblasts. The entire tooth germ showed greater survival capacity toward the periphery of the explant which was exhibited mainly in the epithelium.

Of the 5 specimens sectioned after 12 days in the flask only 2 presented tissue. Both specimens showed the same degree of degeneration. In one case

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Fig. 2.—Nineteen-day rat embryo molar tooth germ cultivated 11 days in embryo extract-free medium photographed in flask. Note degenerated outgrowth and monocyte-like cells. Dark mass (lower left) is original explant. (Orig. mag. ×35.)

Fig. 3.—Nineteen-day rat embryo molar tooth germ cultivated 15 days in embryo extract-free medium photographed in flask. Explant and outgrowth degenerated leaving ring of monocyte-like cells. (Orig. mag. ×35.)

Fig. 4.—Photomicrograph of section of 19-day rat embryo molar tooth germ cultivated 4 days in embryo extract-free medium. Note differentiation of peripheral fibroblasts of papilla. Arrow points to area of degeneration. (Orig. mag. ×100.)

Fig. 5.—Higher magnification proliferative zone of Fig. 4 showing cell division. (Orig. mag. ×450.)

Fig. 6.—Photomicrograph of section of 19-day rat embryo molar tooth germ cultivated 8 days in embryo extract-free medium. Dedifferentiated enamel epithelium, *EE*. Note degeneration of fibroblasts of papilla, *P*. (Orig. mag. ×100.)

Fig. 7.—Photomicrograph of section of 19-day rat embryo molar tooth germ cultivated 12 days in embryo extract-free medium. Dedifferentiated enamel epithelium, *EE*. Note degenerated fibroblasts of papilla, *P*. Arrow points to area of columnar epithelium. (Orig. mag. ×100.)

Fig. 8.—Photomicrograph of section of 19-day rat embryo molar tooth germ cultivated 16 days in embryo extract-free medium. Same specimen as Fig. 1. (Orig. mag. ×100.)

the entire tooth germ was permeated with monocyte-like cells. Fig. 7 is a photomicrograph of a section of 1 specimen, and represents a mass of tissue slightly advanced in degeneration over that observed in Fig. 6. The enamel epithelium shows evidence of dedifferentiation although in 1 isolated area the cells of the inner enamel epithelium retained their columnar form. Degeneration of the papilla was complete.

Four specimens were dissected from the clot after 16 days of cultivation. In each case a small mass of degenerated tissue was recovered. Neither the enamel epithelium nor papilla could be recognized by cytologic identification. In 2 of the specimens a suggestion of the location of the enamel organ aided in identifying the toooth germ. The papilla appeared either as an amorphous or granular mass showing monocyte-like cells in the area. The specimen, which showed the greatest survival capacity, exhibits in Fig. 8 slight evidence of the enamel organ by its location. The papilla presents shreds of acellular tissue. Some fibroblasts of the outgrowth are identifiable even though degenerated.

From gross observations no cultures warranted cultivation beyond 16 days. At this time, observation of the tooth germs in the flask indicated complete degeneration, an observation verified by histologic examination.

DISCUSSION

The in vitro cultivation of tooth germs presents a problem in organized growth, which is concerned with survival, growth, differentiation and the development of functional capabilities. The functional potential of the elements of the tooth germ has been adequately demonstrated although it lags far behind the survival period.

Investigations to date have generally established that the most extensive growth and development occurred in the first days of cultivation.[1-5] In these studies, the use of heterologous media had no apparent ill effect on the explant, and development continued in a harmonious pattern. Survival, and consequently the development of the elements of the rat tooth germ, was seriously hindered after 8 days of cultivation in complete avian media.[3] Using homologous embryo extract and an avian coagulum enhanced survival but the unilateral development of ectodermal and mesodermal derivatives of the organ after 8 days indicates that there is an optimum nutritional potential in the dissected tooth germ at the time of planting.[4]

It seems apparent that the major problem confronting investigators in this area is the nutritional requirement of the explanted organ. Should there be adequate residual optimal nutritional elements in the explant, the evaluation of short-term results should be reconsidered. Furthermore, it becomes necessary to determine the developmental potential of residual media in order to properly interpret findings.

It is difficult to conceive of a nutrient-free medium for the cultivation of tissue. Certainly the coagulum and Tyrode's solution may be construed as nutrients, but embryo extract has been established as the nutritional factor for stimulating growth.[6] Its exclusion would provide a minimal or limited nutritional medium adequate to pursue the investigation of residual nutrient media in the tooth germ at the time of planting.

The survival, growth, and development of a tooth germ in an embryo extract-free medium is an indication that, at the time of planting, it contains a reserve force of nutrition. This may be considered an optimum nutrient residue which provides for the first period of growth and development. In an embryo extract-free medium the maximum growth potential achieved in 4 days of cultivation is equivalent to 1 day of in vivo development. Specimens taken at later periods showed increasing stages of degeneration. Comparing these results with similar periods of cultivation in a medium containing embryo extract, there can be little doubt that embryo extract is a valuable constituent of the nutritional requirements of the tooth germ cells cultivated in vitro and will be essential unless a satisfactory synthetic medium is developed. Survival, growth and development beyond the first 4 days should be attributed to the homologous embryo extract in studies reported to date.

The cells of the enamel epithelium showed greater survival than the fibroblasts of the papilla. The interrelationship of both ectodermic and mesodermic derivatives was disturbed. The dedifferentiation of the enamel epithelium which occurred was similar to that found after the separation of tooth germ elements[10, 11] and in papillectomy[12] which established that the development of the enamel organ is dependent on a normal papilla.

SUMMARY

While no conclusions can be drawn from the limited number of explants, the study suggests that the cells of the enamel organ have greater survival capacity than the fibroblasts of the papilla in embryo and extract-free medium. It is also noted that the tooth germ explant may survive, grow, and develop for 4 days in vitro in the absence of embryo extract. This may explain the first rapid developmental period and establishes the need for embryo extract as a constituent of media in the in vitro cultivation of tooth germs.

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AN INVESTIGATION TO DETERMINE THE EFFECTS OF STARCH SPONGE IMPLANTED IN BONE

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STARCH sponge was discovered accidentally by Scharling in Copenhagen in 1844 when he noted the formation of a spongy mass upon freezing a starch paste. This remained a laboratory curiosity for 100 years until Bice, MacMasters, and Hilbert[1] suggested the use of this material as an antibiotic-carrying medium. Rosenfeld was the first to report on its successful clinical use as an absorbable hemostatic agent.[10-12]

Starch sponge* is a pure cornstarch product in which no other substance is incorporated. It is made by freezing a 5 to 7 per cent paste of cornstarch in water at –8° to –11° C., after which it is thawed at room temperature, cut into desired shapes and sizes, and dried. In the dry state starch sponge can be stored indefinitely if protected from moisture and contaminating organisms. It is hygroscopic and becomes somewhat moist if exposed to air.

The sponge is soft and flexible when wet, hard and rigid when dry. The wet sponge is resilient when pressed, resuming its original shape upon release of the pressure. It will quickly reabsorb all of the moisture pressed from it. The dry sponge rapidly absorbs about 16 times it own weight of water. Sterilization is accomplished in the autoclave.

No significant tissue reaction was observed by Correll, Prentice, and Wise[3] following implantation of starch sponge in rat muscle, although unabsorbed fragments were noted histologically through the fiftieth day. Lee and Lehman,[6] in investigating a number of substances to replace the talc used on surgeons' rubber gloves, found a cornstarch derivative which proved very satisfactory. They showed complete absorption of the cornstarch product from the peritoneum without demonstrable inflammatory reaction and without producing adhesions. MacQuiddy and Tollman[7] reported on the satisfactory use of a cornstarch derivative, treated by physical and chemical means to improve its lubricating value and to prevent gelatinization when autoclaved, to replace talc. They were unable to find any sensitivity to this starch in the group of human beings tested. The cornstarch derivative was nonirritating in the tissue of animals and was readily absorbed from the peritoneal cavity. Rosenfeld[10-12] found starch sponge to be an excellent hemostatic agent in the rabbit. The peritoneal

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*Manufactured under the trade name of "Solusponge" by the Panray Corporation operating under a patent license granted by the Department of Agriculture and through the courtesy of whom the material used for this investigation was donated.

adhesions found at autopsy performed on these animals 3 to 4 weeks after implantation of the sponge were described as "surprisingly moderate." Clinically the sponge was successfully used to control bleeding in obstetrics and in gynecologic surgery. The hemostatic effect was attributed to mechanical action, swelling, and consequent pressure, rather than any acceleration of the clotting mechanism. Ricchiuti[8] demonstrated complete absorption of the sponge from the peritoneal cavity of dogs in 24 hours and from subcutaneous implant in 72 hours. Ritter and Bloomberg[9] found starch sponge especially suitable to prostatic surgery. In a series of 687 cases Davidson and Sedell[4] found starch sponge superior to previous products used in extraction sockets for hemostasis. The incidence of dry sockets did not increase following the prophylactic use of this material.

The purpose of this investigation was to determine the biologic absorbability of starch sponge in bone, the histologic response that might be provoked by implantation of starch sponge in bone, the effect upon healing following implantation of starch sponge in the tooth extraction socket and the incidence of alveolar osteitis following implantation of starch sponge in the tooth extraction socket. The first 3 phases of the investigation were carried out in dogs, the fourth in humans.

EXPERIMENTAL PROCEDURE

In dogs the starch sponge was implanted in the mesial socket of the upper fourth premolar, the distal socket remaining as a control, and in the ilium on the lateral aspect below the crest. Intravenous Nembutal anesthesia was employed for all procedures on the dog and sterile technique observed for the operation on the ilium.

The dogs were sacrificed so that specimens were obtained that had been embedded for 1, 2, 3, 4, 5, 7, 10, 14, 21, and 28 days. All specimens were radiographed and placed in a 10 per cent formalin solution. They were subsequently decalcified in formic acid and blocked in wax for sectioning. Staining was done with hematoxylin and eosin, and periodic acid-Schiff to detect the presence of carbohydrates.

For the investigation in human subjects sterile starch sponge cut to size for insertion into the tooth socket was supplied in cotton-stoppered vials. The clinicians participating in the investigation were instructed to use the sponge dry, without adjuncts, in the tooth sockets of patients, without selection, presenting for treatment during the time of the investigation. The only exception was in the case of the suppurative socket where the use of starch sponge is contraindicated.[4] The patients were instructed to report 1 week postoperatively for examination.

RESULTS

A. *Animal Experimentation.*—Radiologic changes in the tooth socket did not become evident until between the second and third week. At that time the radiograph gave evidence of osteogenesis, farther advanced in the distal socket (control) than in the mesial socket (starch sponge).

Fig. 1.

Fig. 2.

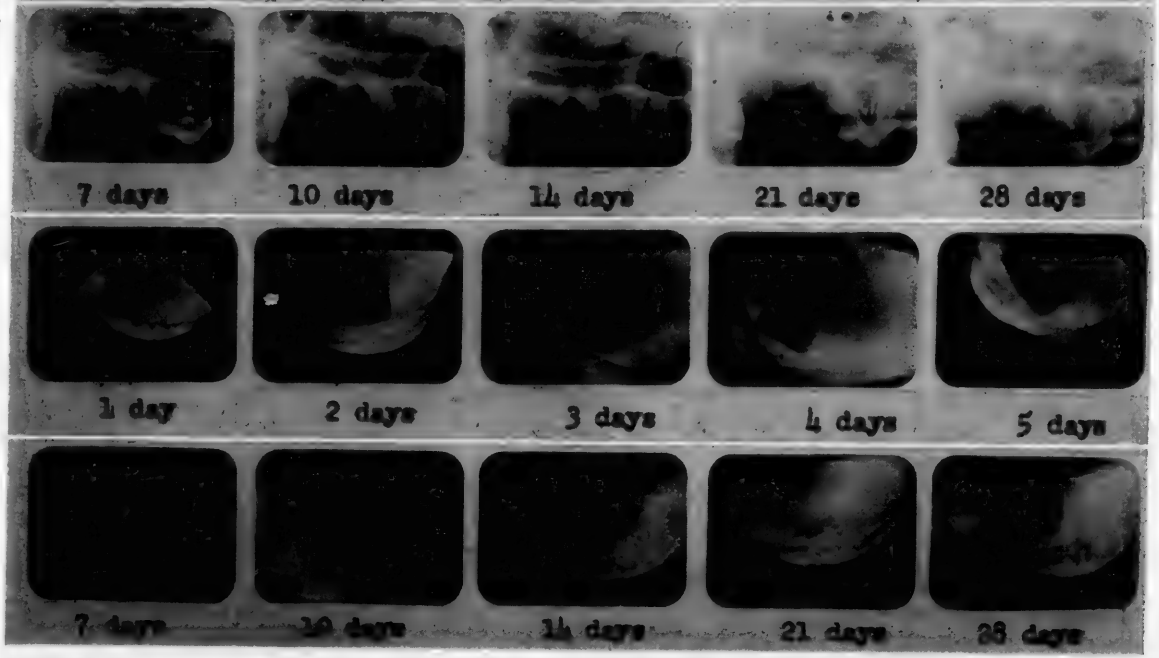

Fig. 1.—Radiographs of fourth premolar tooth sockets used in the investigation. Osteogenesis is evident in the 21-day specimen, farther advanced in the control socket than in the starch-sponge-implanted socket.

Fig. 2.—Radiographs of the ilia used in the investigation. Osteogenesis is evident in the 21-day specimen.

An identical pattern was observed in the ilium. The abrupt change between the second and third week was clearly demonstrated (Figs. 1 and 2). The apparent loss of density in the 28-day ilium specimen was due to a through-and-through wound of both cortices which produced the circumscribed area of radiolucence present in the center of the bone defect. Histologically this central defect appeared as a dense collagenous mass.

Fig. 3.

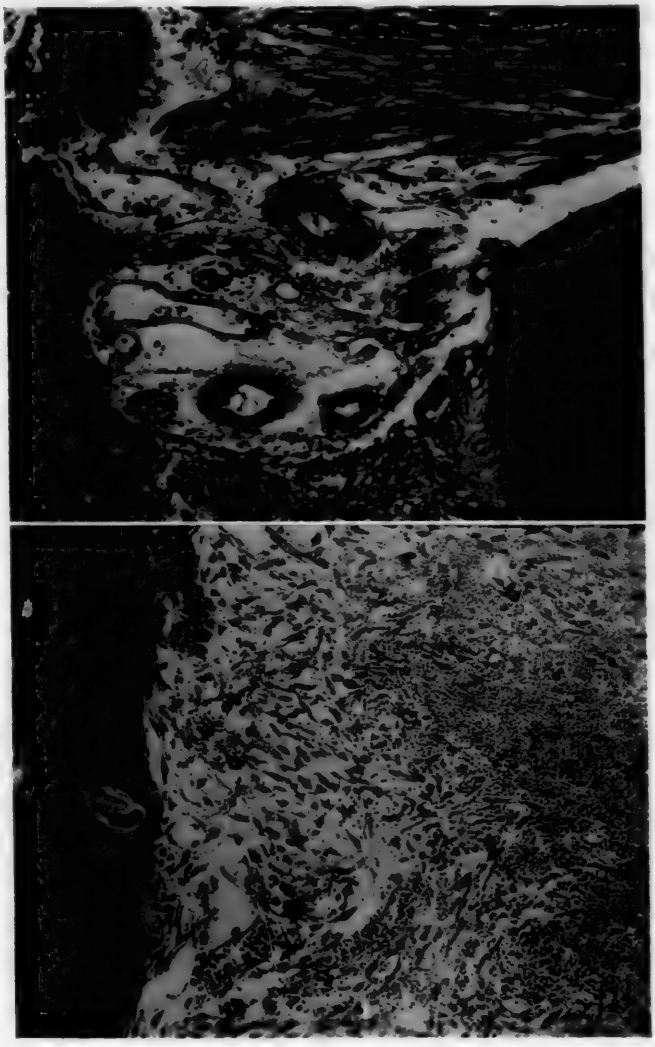

Fig. 4.

Fig. 3.—Two-day control tooth socket. Beginning proliferation of fibrous tissue from the sides of the wound. (Hematoxylin and eosin; orig. mag. ×145.)

Fig. 4.—Four-day control tooth socket. Proliferation of granulation tissue is very active from sides of wound. Remodeling of bone is occurring along sides of wound. (Hematoxylin and eosin; orig. mag. ×145.)

In dogs the control tooth socket demonstrated normal healing and the histological pattern followed closely that observed by other investigators.[2, 5] Beginning proliferation of fibrous tissue from the sides of the wound was evident in two days (Fig. 3).* In 4 days the proliferation of granulation tissue was

Fig. 5.

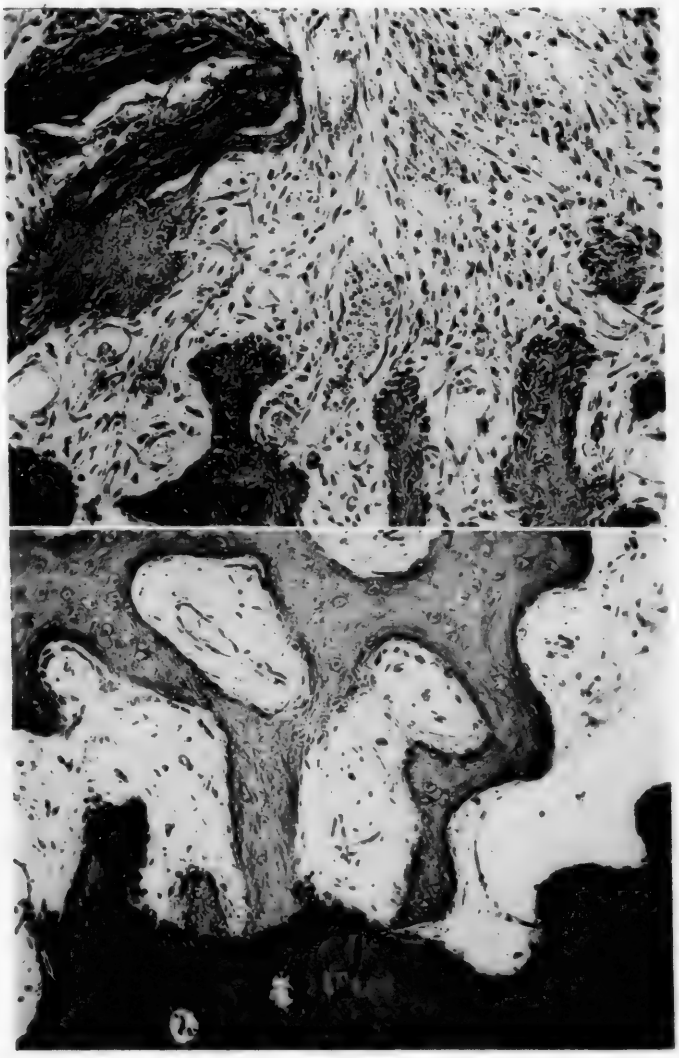

Fig. 6.

Fig. 5.—Seven-day control tooth socket. Osteogenesis is proceeding rapidly. (Hematoxylin and eosin; orig. mag. ×145.)

Fig. 6.—Twenty-one-day control tooth socket. Newly formed bone completely fills the bone defect. (Hematoxylin and eosin; orig. mag. ×145.)

*Photomicrographs through the courtesy of the Medical Illustration Service of the Armed Forces Institute of Pathology, Washington, D. C.

very active (Fig. 4). In 7 days osteogenesis was proceeding rapidly (Fig. 5) and in 21 days newly formed bone completely filled the bone defect (Fig. 6).

The tooth socket in which starch sponge had been implanted demonstrated delayed healing. In 1 day there appeared to be an acute inflammatory reaction

Fig. 7.

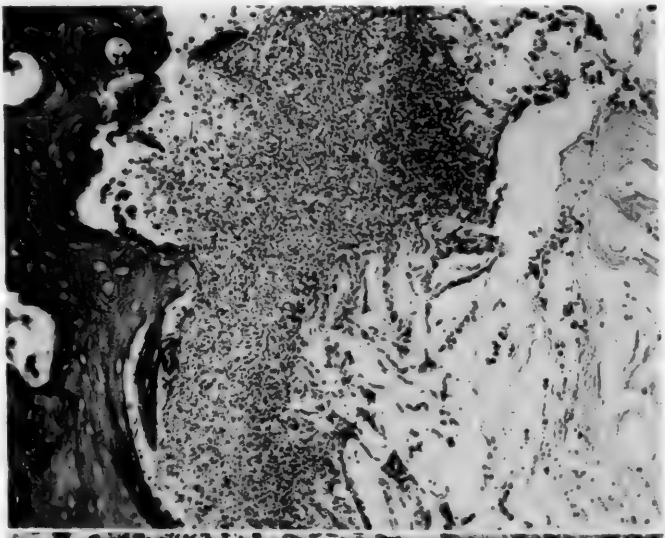

Fig. 8.

Fig. 7.—One-day starch-sponge-implanted tooth socket. There is an acute inflammatory reaction surrounding the implanted sponge with polymorphonuclear neutrophils predominating and infiltrating the sponge. (Hematoxylin and eosin; orig. mag. ×145.)

Fig. 8.—Three-day starch-sponge-implanted tooth socket. There is an active fibroblastic and endothelial proliferation from the side of the tooth socket accompanied by mononuclear cells and polymorphonuclear neutrophils converging on the blood clot residue. (Hematoxylin and eosin; orig. mag. ×145.)

surrounding the implanted sponge with polymorphonuclear neutrophiles predominating and infiltrating the sponge (Fig. 7). In 2 days fibrous proliferation from the sides of the wound was evident, but the material remaining in the socket appeared to be a necrotic mass. In 3 days fibrous and endothelial

Fig. 9.

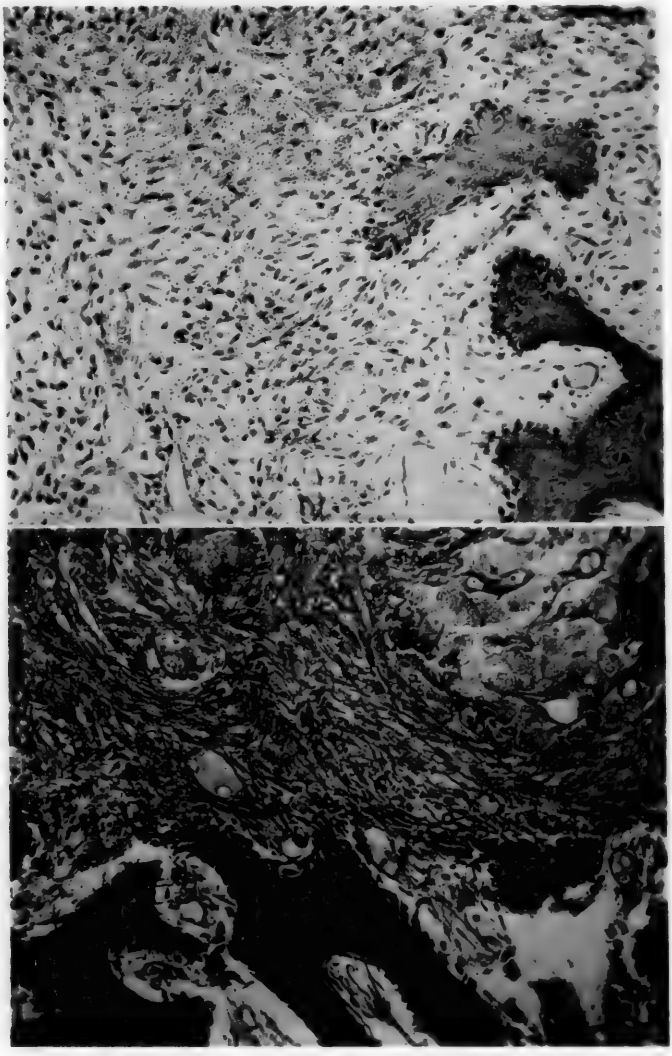

Fig. 10.

Fig. 9.—Seven-day starch-sponge-implanted tooth socket. Granulation tissue is proliferating from the sides of the wound and osteogenesis is occurring. (Hematoxylin and eosin; orig. mag. ×145.)

Fig. 10.—Twenty-one-day starch-sponge-implanted tooth socket. Newly formed bone completely circumscribes the healing socket. Granulation tissue is still present in the center of the tooth socket. (Hematoxylin and eosin; ×145.)

proliferation were very active (Fig. 8) but it was not until 7 days that the starch-sponge-implanted tooth socket approached the 7-day control. Here osteogenesis was evident, although not quite as advanced as the control (Fig. 9). At 21 days granulation tissue was still present in the center of the starch-sponge-implanted tooth socket (Fig. 10), and it was not until the twenty-eighth day that the sockets appeared identical histologically.

Fig. 11.

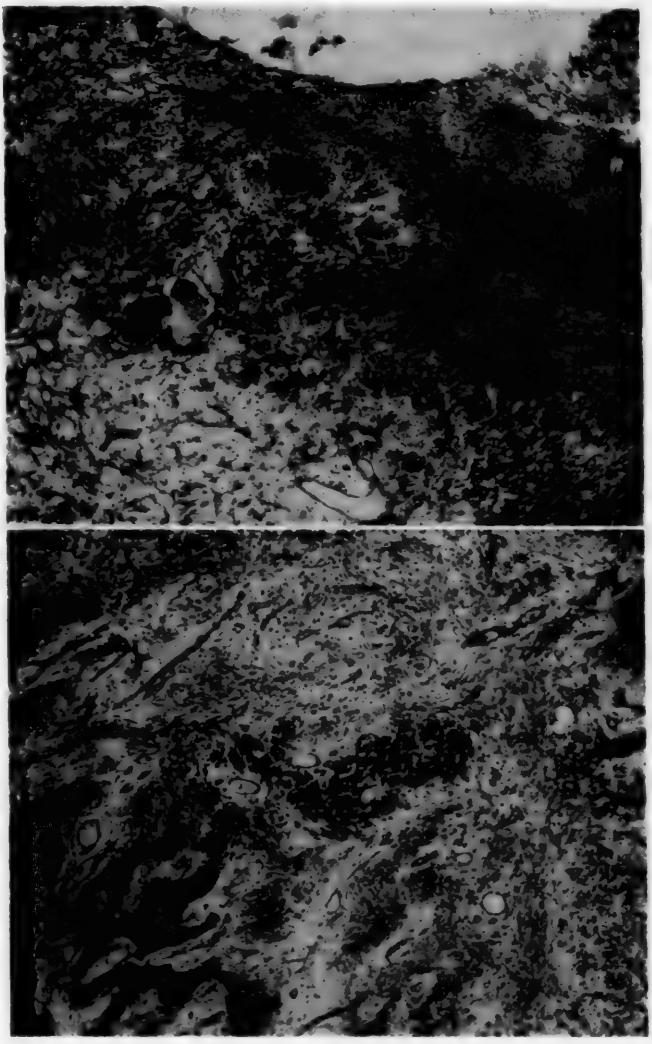

Fig. 12.

Fig. 11.—Three-day starch-sponge-implanted tooth socket. Fragment of starch sponge at fundus of socket. (Periodic acid-Schiff; orig. mag. ×65.)

Fig. 12.—Ten-day starch-sponge-implanted tooth socket. Residual granules of starch sponge. (Periodic acid-Schiff; orig. mag. ×65.)

Using the periodic acid-Schiff stain, a stain for carbohydrates, fragments were seen as late as 28 days after implantation. Hydrolysis of starch sponge in the tooth socket did not proceed with any regularity and could not be attributed to physiological reactions but appeared to be the result of the inflammatory process (Figs. 11, 12 and 13).

Healing in the ilium followed a normal pattern and closely paralleled the healing in the control tooth socket. The implanted starch sponge provided a well-tolerated matrix for the blood clot and appeared to be absorbed at the same rate as the blood clot. This was well demonstrated in the specimen in which starch sponge had been embedded for 10 days. Here the starch sponge was absent at the borders of the wound where osteogenesis was active, yet was present deeper in the wound where granulation had not yet occurred.

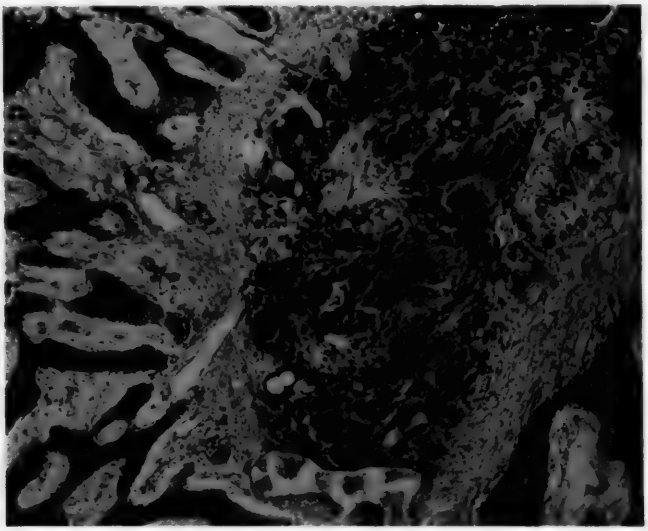

Fig. 13.—Twenty-one-day starch-sponge-implanted tooth socket. Residual fragments of starch sponge in center of tooth socket. (Periodic acid-Schiff; orig. mag. ×65.)

Using the periodic acid-Schiff stain in the ilium, it was noted that although the strands of starch sponge were absorbed as granulation proceeded, numerous residual granules remained unabsorbed and embedded in the granulation tissue and newly formed bone marrow. These granules, present as inert bodies, did not elicit any unusual cellular response during the time of this investigation. Further study is necessary to determine the final reaction of the organism to these residual fragments.

B. *Clinical Findings.*—Eleven hundred and fifty-two extraction sockets were examined in 528 patients. Three hundred and sixty-five sockets were implanted with starch sponge, of which 229 were checked postoperatively. The remainder were not checked because the patients did not return. There were 7 cases of alveolar osteitis (1.92 per cent).

Seven hundred and eighty-seven sockets served as controls, 457 of which were checked postoperatively. The remainder were not checked because the patients did not return. There were 6 cases of alveolar osteitis (0.76 per cent). The chi square was computed to be 2.98.

DISCUSSION

A. *Biologic Absorbability in Bone.*—In the bone wound produced experimentally in the crest of the ilium, starch sponge appeared to be absorbed at the same rate as the blood clot. This suggests that the mechanism of hydrolysis of starch sponge in bone might in part be due to starch-splitting enzymes released by the proliferating cells. It is also possible that the exudate from the newly formed vascular channels contains sufficient amylase to hydrolyze the starch. Doubtlessly some of the material is removed by phagocytosis.

Notable is the fact, however, that following the hydrolysis of starch sponge strands, residual granules, which are resistant to hydrolysis, remain. These, unlike the starch sponge strands, were not identified readily when examined under the hematoxylin and eosin stain but were clearly observed when the periodic acid-Schiff stain was used. The difference in staining reactions indicates that this material is not cornstarch. The positive identification of this material, however, is beyond the scope of this investigation.

B. *Histologic Response in Bone.*—The acute inflammatory response that accompanied the presence of starch sponge in the dog tooth socket can be attributed to the growth of organisms in the culture medium produced by the action of ptyalin on starch. It was felt that the starch might have been hydrolyzed in the autoclave or by starch-splitting organisms present in the oral flora. The autoclaved starch sponge was tested for sugar with negative results. The existence of starch splitting microorganisms among the oral flora has not been determined.

In the ilium the histologic response was that which would be anticipated in a healing bone wound. There was no unusual histologic response. Some of the residual granular material was present in phagocytic cells but the bulk of the material remained inert, apparently well tolerated.

C. *Effect Upon Healing.*—The histological sections demonstrated retarded healing in the starch-sponge-implanted tooth socket. This cannot be attributed to the action of starch sponge per se, but to secondary bacterial invasion which followed the degradation of starch into sugar. One of the factors which might have influenced this reaction was the size of the implanted sponge. In the experimental animals the implanted sponge filled the socket completely. If a smaller volume of sponge had been implanted in the apical one-third of the tooth socket, it is possible that the mouth fluids would not have been able to reach the sponge to produce hydrolysis.

Clinical observations made on the dogs at the time they were sacrificed demonstrated delayed epithelization as a consistent finding in the starch-sponge-implanted tooth sockets.

Healing in the bone cavity produced under sterile conditions in the ilium followed a pattern that was remarkably similar to the control tooth socket. The implanted sponge appeared to be compatible with the surrounding tissue. It has not been observed that starch sponge acts as a network upon which fibrosis occurs but rather that the sponge becomes an integral part of the blood clot, absorbed with the clot as fibrosis proceeds. The residual granules, noted throughout the investigation, were present as inert bodies and did not appear to have any effect upon healing.

D. *Incidence of Alveolar Osteitis.*—The total number of extraction sockets was used in the computation of the chi square even though some patients did not return for postoperative examination. All patients were instructed to report 1 week postoperatively, or sooner if any untoward symptoms developed. Since the subjective symptoms of alveolar osteitis are so distressing, it was felt that patients who developed this condition would return for treatment. It was assumed that those patients who did not return did not develop alveolar osteitis, and the inclusion, therefore, of all patients in the statistical analysis was justified.

The chances of obtaining a chi square value of 2.98 due to sampling error are between 5 and 10 in 100. This might be considered the outer limit of significance. From this and the results of animal experimentation, one can conclude that while the use of starch sponge in the extraction socket does not increase the incidence of osteitis alveolar significantly, it predisposes to postoperative infection and delayed healing.

CONCLUSIONS

In the bone wound produced under sterile conditions, starch sponge is absorbed at approximately the same rate as the blood clot, leaving residual granules at 28 days, the length of time of this investigation. Starch sponge does not appear to provoke any unusual histologic response and does not interfere with the rate of osteogenesis.

In the contaminated bone wound the absorption of starch sponge is not accomplished in a physiological manner but rather accompanying inflammation. Implantation of starch sponge predisposes to infection and the cellular response is that of inflammation. Due to the secondary infection which follows the implantation of starch sponge in the tooth socket, healing is retarded.

Implantation of starch sponge in the tooth extraction socket does not significantly increase the incidence of alveolar osteitis but it does predispose to delayed healing.

SUMMARY

The present investigation was undertaken to determine the effects of starch sponge in bone. Dogs were used as experimental animals and starch sponge was implanted in the tooth socket and ilium. The clinical phase of the investigation was carried out in humans and consisted of a determination of the incidence of alveolar osteitis following implantation of the sponge.

It was determined that starch sponge does not interfere with osteogenesis but residual granules remain in the newly formed bone marrow. Due presumably to degradation followed by secondary infection, its use in the dog tooth socket is accompanied by an acute inflammatory reaction with resultant delay in healing. When used in the human tooth socket, the incidence of alveolar osteitis does not increase significantly, but healing is retarded.

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RESIDUAL MERCURY CONTENT OF AMALGAM RESTORATIONS AND ITS INFLUENCE ON COMPRESSIVE STRENGTH

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IT HAS long been thought that the residual mercury content of amalgam restoration does influence, to some extent, both the compressive strength and resistance to tarnish. Ward and Scott,[1] investigating primarily the effects of packing pressures on dimensional change, made some observations on compressive strength as related to residual mercury. They noted that when the packing pressures were increased, there was a trend toward higher compressive strengths and lower residual mercury contents. The use of extremely high condensation pressures (256,000 psi) reduced the residual mercury to 28.3 per cent and the compressive strength rose to 73,000 psi. Such pressures are, of course, not possible in the clinical insertion of an amalgam restoration.

Gray's[2] original work illustrated that as the mercury decreased below 50 per cent the strength increased. Crawford and Larson[3] demonstrated that the compressive strength of amalgam increased from 53,000 psi to approximately 59,000 psi when the residual mercury fell from 48 to 35 per cent. However, they found no correlation between strength and residual mercury within limits of 45 to 50 per cent. Likewise Ryge, Dickson, Smith, and Schoonover,[4] were unable to demonstrate any relationship in strength with specimens where the residual mercury ranged from 46.5 to 53.0 per cent. Swartz and Phillips[5] detected no influence upon compressive strength of residual mercury between limits of 45.6 and 48.5 per cent.

The studies cited were designed primarily to investigate the effect upon compressive strength of minimal amounts of mercury which might be secured by use of heavy condensation pressures. As Crawford has summarized,[3] there seems to be little gained, at least in terms of strength, in the development or use of a technic which would reduce the mercury content below 45 per cent.

Little work has been done, however, to correlate the strength with the residual mercury at the higher levels that are more commonly found in the amalgam restoration. Analyses of amalgam restorations removed from extracted teeth indicate that mercury is often present in amounts well above 50 per cent. Crawford and Larson[3] analyzed 10 restorations and found the residual mercury content ranged from 45.2 to 59 per cent. Souder and Sweeney[6] analyzed 2 restorations and found 51.6 per cent mercury in one, and 52.9 per cent

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in the other. Phillips and Swartz,[7] reporting on 100 restorations selected at random, found mercury to be present in widely varying quantities from 28.6 to 61.0 per cent. It is interesting to note that in their investigation a large number of restorations contained mercury in amounts that exceeded 50 per cent. It must also be pointed out that in all these studies, the entire specimen was analyzed. It is quite possible that certain specific areas might contain significantly higher values of residual mercury than the mean for the whole sample.

Thus, although there are ample data correlating strength with residual mercury at the lower levels, no work has been done to study the influence played at the higher concentrations which are often found in the entire clinical restoration or in some specific area. This study was designed, therefore, to determine the effects on the compressive strength of residual mercury when it exceeded 50 per cent and to measure the mercury content in various areas of amalgam restorations, particularly the margins where fraying and fracture are often evidenced.

PROCEDURE

Method of Mercury Analysis.—The method of mercury analysis employed throughout the study was that of Crawford and Larson.[8] In this procedure, the mercury is volatilized, from a pulverized sample of amalgam, in an inert gas atmosphere and the residual mercury determined by the loss in weight of the specimen.

Crawford and Larson employed relatively large powdered specimens in their study. However, analyses of different parts of restorations, especially marginal areas, would necessitate measurements being made on extremely small quantities of alloy. Where such small samples are involved, the possible use of whole pieces could minimize loss of some of the amalgam when the sample is being pulverized. Thus an experiment was conducted to determine: (1) The accuracy of the method when small samples are employed. (2) Whether the mercury content of whole pieces of amalgam could be determined as accurately as pulverized samples.

Amalgam specimens of known mercury content were prepared by weighing the mercury and alloy, in a mortar, on an analytical balance. They were then carefully triturated to incorporate all of the alloy and mercury and to produce, as nearly as possible, a homogeneous mix. For the pulverized samples, the entire mass was powdered in a diamond mortar and pestle and sample aliquots taken. Whole samples were prepared merely by cracking the entire mass, and pieces of varying size were then selected for analysis.

Fifty pulverized samples of amalgam of known mercury contents, varying in size from 417 mg. to 8 mg., were analyzed. The residual mercury in another additional 50 whole pieces of amalgam, ranging in size from 348 mg. to 3 mg., was also determined.

No significant difference was found in the accuracy of the method with either pulverized or whole samples of amalgam. The standard error in the

analysis of whole pieces was ±0.10 per cent or ±0.09 mg. while that of pulverized samples was ±0.07 per cent or ±0.06 mg. Likewise whole pieces and pulverized samples of amalgam weighing 25 mg. or less were analyzed with the same precision as those weighing 100 mg. or more. On the basis of these results it was felt that it was possible to measure samples as small as 10 mg., either whole or pulverized.

Analyses of Various Areas of Amalgam Restorations.—In the restorations to be analyzed for residual mercury, care was taken to select those which could be considered average or good. Restorations which showed badly frayed margins, improper contour, etc., were not analyzed. The amalgam was removed from the extracted teeth, cleaned of debris, and the specific areas to be analyzed were cut from the restoration. The remainder of the amalgam was then pulverized in a diamond mortar and pestle and samples taken for analysis. Thus it was possible to compare the mean mercury content of the entire restoration with that of certain specific areas. A diagram indicating the approximate areas analyzed can be seen in Fig. 1. These particular areas were arbitrarily selected.

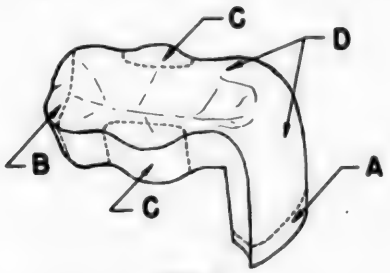

Fig. 1.

The mercury contents in the various portions of 17 restoration are given in Table I. The fact that an amalgam restoration is not usually homogeneous is readily evident from these analyses. The amount of mercury present in different portions of the same restoration varied as much as 6 per cent.

TABLE I

RESTORATION NO.	% Hg			
	A	B	C	D
1	55.2	59.0	58.2	56.7
2	57.2		58.3	59.3
3	54.6	55.3	55.3	54.3
4	56.3	54.3	55.1	54.6
5	47.9	52.2	51.8	49.1
6	59.0	62.5	57.5	57.1
7	50.5	53.1	53.7	51.7
8	54.7	59.9	57.4	56.3
9	54.1	51.1	48.5	53.4
10	49.7	46.3	51.9	50.4
11	44.0	49.9	50.0	48.9
12	49.4	51.1	50.9	51.1
13	50.3	50.2	53.5	51.3
14	51.1	52.0	53.6	50.3
15	51.9	50.5	52.0	50.7
16	55.4	51.9		52.9
17	57.3	58.6	58.5	60.2

TABLE II

RESTORATION NO.	% Hg AT MARGINS	% Hg IN BULK
1	54.7	52.4
2	58.6	58.6
3	58.8	56.5
4	56.5	55.0
5	53.0	56.8
6	49.5	49.7
7	55.8	53.6
8	58.7	60.4
9	60.8	60.3
10	55.4	54.1

Ten additional restorations were removed from extracted teeth and the entire marginal area, approximately 0.5 mm., was ground with a slow moving bur, collected and analyzed. The residual mercury content at the margins, as compared to the bulk of the restoration, can be seen in Table II. Again it is apparent that the residual mercury at the margins may vary considerably from that present in the bulk of the restoration.

Analyses of these random restorations clearly indicate that residual mercury content is often high. Values above 55 per cent were quite common. Since no one has correlated the compressive strength with residual mercury at these levels, the following series of tests were made.

Compressive Strength as Related to Residual Mercury.—In the preparation of compressive strength specimens, the same batch of a representative, small grained alloy was used throughout. A Crandall balance was employed as the proportioning device and all trituration was done by means of a mechanical amalgamator. No mercury was expressed following trituration; the entire mass was condensed immediately into a 5 mm. by 10 mm. mold by means of an automatic packing device. The load was applied for 2 minutes. The compressive strength tests were run on a Tinius Olsen machine, the load being applied at the rate of 9,200 psi per minute. Specimens were tested for strength both at 1 and 24 hours and the fractured sample then analyzed for residual mercury content. The figures shown are the mean values obtained from a minimum of 6 and a maximum of 12 specimens.

It has been suggested that there are factors other than the residual mercury content which might influence the compressive strength of amalgam. For example, if excessive condensing pressures are employed, although more mercury is removed it is possible that the closer adaptation of the alloy particles and reduction of any internal voids produces a more dense mass and increases the strength. In order to determine whether residual mercury, per se, was the lone factor governing compressive strength, groups of specimens were prepared in which condensation pressures of 500, 1,000 and 2,000 psi were used. Thus, it was possible to compare the strength of specimens which contained approximately the same residual mercury but which had been condensed with varying pressures.

In order to prepare specimens which had different residual mercury contents, various original mercury-alloy ratios were employed. (When the condensation pressure was 2,000 psi, it was necessary to exaggerate the original

ratio to secure high residual mercury in the final specimens.) This procedure proved to be satisfactory and for a given condensation pressure and mercury-alloy ratio, the residual mercury content in the condensed specimen was quite uniform. In Table III the residual mercury in specimens prepared from 3 different mercury-alloy ratios is shown. The results were reproducible.

TABLE III

SPECIMEN NO.	5:8 RATIO	5:15 RATIO	5:30 RATIO
1	54.0%	54.9%	57.4%
2	54.1%	55.5%	57.1%
3	53.9%	55.2%	57.0%
4	54.0%	55.6%	57.0%
5	54.2%	54.8%	57.1%
6	53.7%	54.9%	56.9%

2,000 lb./sq. in.

The residual mercury content of specimens prepared with various mercury-alloy ratios and condensed with the 3 different pressures are shown in Fig. 2.

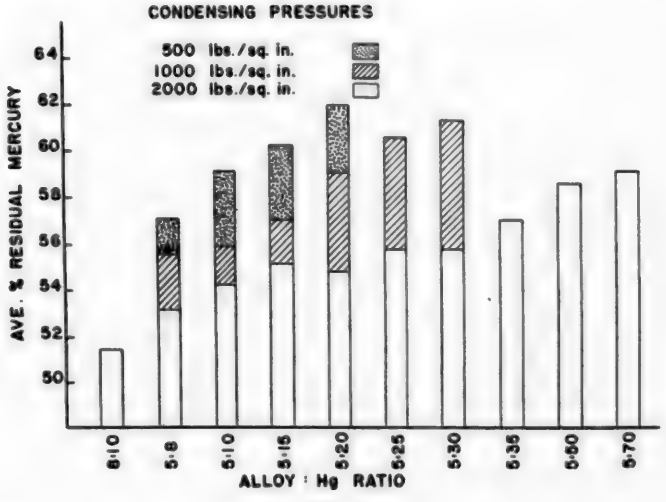

Fig. 2.

For any given condensation pressure, increasing the percentage of mercury in the original mix was reflected in higher residual mercury contents in the final specimen. Specimens condensed at 2,000 psi from a mix of amalgam containing 8 parts mercury to 10 parts alloy resulted in an average 51.3 per cent residual mercury, while those prepared from mixes utilizing 25 parts mercury to 5 parts alloy condensed at the same pressure, contained an average of 55.9 per cent residual mercury. This corroborates previous work by Phillips and Boyd[9] that the original mercury-alloy ratio does influence the residual mercury content, irrespective of the condensation pressure.

For each individual mercury-alloy ratio where more than one condensing pressure was employed, it will be noted that mercury content rose as the condensing pressure decreased. The average residual mercury content of amalgams prepared from mixes where an alloy-mercury ratio of 5:8 was used in conjunction with only a 500 psi packing pressure was as great as that of specimens where more than 4 times as much mercury (5:35) were employed in the original mix but condensed at the higher pressure of 2,000 psi. Thus previous work[2, 3] showing that mercury content is inversely related to condensation pressure is substantiated. It is obvious that if use of excess mercury in the original mix is accompanied by inadequate condensation pressure, the residual mercury can reach extremely high values.

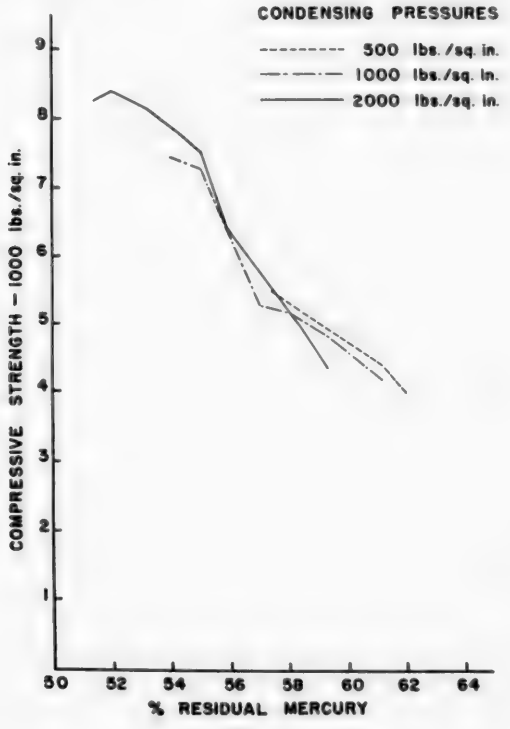

Fig. 3.

The 1 hour compressive strength of specimens with various residual mercury contents is shown in Fig. 3. It can be seen that generally as the mercury content increases the compressive strength decreases, particularly when the residual mercury exceeds 55 per cent. It was not possible to prepare specimens of low residual mercury when condensation pressures of 500 and 1,000 psi were used since the packing pressures were not adequate to remove sufficient quantities of mercury.

Twenty-four-hour compressive strength specimens also showed a decrease in strength as the residual mercury contents rose (Fig. 4). It is of interest to note that the strength was quite constant until the residual mercury exceeded approximately 55 per cent. From that point on, however, the strength was rapidly dissipated as the mercury content increased. When a condensation pressure of 2,000 psi was used, the strength which was 40,000 psi with a residual mercury content of 55 per cent dropped to only 18,000 psi when the mercury content rose to 59 per cent. With the low condensation pressure of 500 pounds per square inch, it was possible to prepare specimens containing as much as 62 per cent residual mercury and these produced strengths of only 14,000 psi.

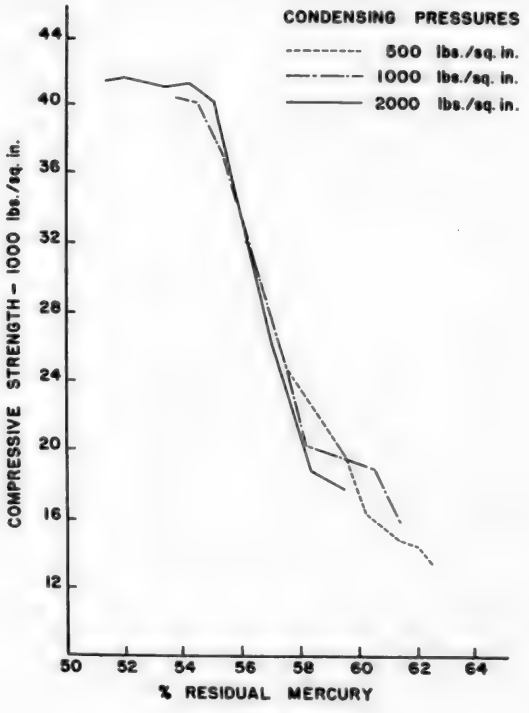

Fig. 4.

An analysis of these data clearly indicates that for this alloy above 55 per cent there is a direct relationship between residual mercury content and compressive strength. Specimens prepared at various condensation pressures but having the same residual mercury content invariably yielded approximately the same compressive strength. For example, when the residual mercury was in the range of 57.0-57.5 per cent, the variation in compressive strength of specimens prepared by 3 different condensation pressures was only 2,000 psi.

In order to check these data another alloy was also tested. One with a larger grain size was employed and specimens prepared as in the previous work. Since comparable results were obtained the data have not been reported in this paper.

DISCUSSION

In spite of the fact that amalgam restorations are inserted which contain residual mercury contents approaching 60 per cent, in this study with the condensation pressures which were employed it was necessary to increase the amount of mercury in the original mix to most exaggerated proportions in order to obtain the desired high concentrations of residual mercury in the condensed specimens. This was not surprising in view of observations made by Crawford and Larson,[3] who found that when the recommended mercury-alloy ratios were employed, the residual mercury content of amalgam which was just jarred into the mold was approximately 60 per cent. The results of this investigation indicate that the residual mercury content is principally controlled by 2 factors, the original mercury-alloy ratio and the condensing pressure. When high concentrations of residual mercury are encountered clinically, it suggests use of either an improper mercury-alloy ratio, insufficient condensation pressure or perhaps a combination of the two.

Earlier studies[1-3] indicated that use of extremely high condensation pressures substantially reduced the residual mercury content but there seemed to be no correlation between strength and residual mercury when it was within the range of 45 to 53 per cent.[4, 5] The data obtained in this investigation indicate that mercury content between these limits is not critical and that the compressive strength is not impaired until the residual mercury reaches 55 per cent. For these alloys above 55 per cent there seems to be a direct relationship between strength and residual mercury. This particular percentage appears to be critical since both alloys tested maintained a relatively constant compressive strength when the residual mercury was below this figure, but displayed a dramatic loss in strength when it was exceeded. It must be recognized that this critical value may vary with the alloy used.

The analyses of different areas of amalgam restorations indicate that although the average residual mercury content of the entire restoration may be less than 55 per cent it is quite common to find certain areas which may exceed that figure. Certainly those areas of high mercury, and hence low strength, would be more vulnerable to fracture if subjected to stress. Care must be exerted in adhering to the recommended ratio and using adequate condensation pressure and technic to minimize residual mercury and possible fracture.

SUMMARY

It has been shown that the method employed here for mercury analysis of amalgam alloys is accurate for use with small and unpulverized specimens. Analyses of 27 amalgam restorations indicated that they are not homogeneous and that residual mercury in some critical areas often approaches 60 per cent. Residual mercury does vary with the original ratio and with the condensation pressure. With the two alloys used, although compressive strength was not altered appreciably by fluctuations in residual mercury content between the ranges of 50-55 per cent, there was a serious loss in strength when the mercury exceeded 55 per cent.

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THE EFFECT OF LYMPHOCYTIC CHORIOMENINGITIS ON PREGNANCY AND DENTAL TISSUES IN MICE

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ALTHOUGH based primarily upon clinical evidence, the relationship of congenital abnormalities to prenatal disease, whether metabolic or infectious, has been established.[1-3] On the other hand, little information is available on the specific subject of prenatal influences on tooth development. That such a relationship is perhaps uncommon is evidenced by the apparent rarity of defects in deciduous teeth.[4, 5] However, since this contention is supported almost entirely by clinical observation and neglects the important consideration of histologic study, the question remains a moot one.

Most studies have been necessarily conjectural in their evaluation of the placenta as a barrier to or pathway for agents that might be causally related to congenital abnormalities. It has been suggested that such virus diseases as rubella, rubeola, mumps, varicella, and herpes zoster are possibly of etiologic significance,[6-8] but the mechanism by which they may initiate fetal defects is not established. Consideration must necessarily be given to the possibility of metabolic by-products of the maternal disease as well as actual transplacental infection. In either case, conclusive information has not been forthcoming from experimental studies due to the limited host range of these viruses. In order to further explore the question of prenatal influences on tooth development, it became necessary to consider other viruses that are pathogenic for the more common laboratory animals of choice. Unfortunately, most of these have not been demonstrated to be capable of placental passage. A previous study[9] of vaccinia failed to confirm findings of others relative to intrauterine fetal infection. Consistent with the evidence for an apparent placental barrier to vaccinia virus was the finding of severe dental defects in the maternal rabbits and the absence of dental abnormalities in the young.

It became of interest to pursue a similar direction of study with a virus definitely known to be capable of transfer from maternal to fetal circulation. As demonstrated by Traub,[12] lymphocytic choriomeningitis in mice satisfies this requirement. It was further reported by Haas[13] that mice infected in utero retained active virus for long periods and, in turn, transmitted the infection to their offspring. There thus seems to be ample evidence that such congenitally acquired infection results in a prolonged viremia. On the other hand, inoculation of mature mice is followed by a rather short period of viremia and a consequent inability to transmit infection to offspring unless such inoculation is

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accomplished shortly before or during pregnancy. Although the virus used is neurotropic, intraperitoneal inoculation in mice has been observed to cause a variety of tissue changes in the lung, liver, kidney, spleen, lymph nodes, bone marrow, adrenals, pancreas, salivary glands, and other structures.[14]

Although developmental dental abnormalities are undoubtedly related to a wide variety of injurious agents, the possibility of any one selected virus in the present series of studies being etiologically related to such defects is a matter of considerable chance. A similar element of chance, although culminating in the demonstration of positive maternal findings, was apparent in our previous study of vaccinia infection.

MATERIALS AND METHODS

This report is based upon findings in 203 female Swiss mice selected from our established colony on the basis of age (2 to 4 months) and demonstrated evidence of successful breeding. In all instances, the appearance of a vaginal plug after exposure to males of known fertility was used as a criterion for dating the onset of gestation. One hundred and fifty-three of the animals were inoculated intraperitoneally with the virus of lymphocytic choriomeningitis. Twenty others were similarly inoculated with a heat-killed suspension of virus, and the remaining 30 mice served as breeding controls.

In order to establish a source of virus, 5 congenitally infected mice were obtained from the National Institutes of Health.* Normal mice, aged 6 to 8 weeks, were then inoculated intracerebrally with a suspension of blood from this infected stock. Following the appearance of characteristic signs of lymphocytic choriomeningitis, usually evident at 6 to 8 days, the animals were sacrificed and their aseptically removed brains stored in 60 per cent neutral glycerol at 4° C. Then, as needed for inoculation of pregnant animals, the macerated brain tissue was suspended in saline and diluted to 10^{-3}. Dosages of 0.25 c.c. of this dilution were administered intraperitoneally to each of 153 bred mice. This dose was chosen on the basis of its demonstrated ability to initiate a clinically manifested disease with low mortality. Sixty-eight of the animals were inoculated during the first trimester of pregnancy (1 to 6 days), 63 during the second trimester (7 to 13 days), and 22 during the terminal week of pregnancy (14 days to term). In all cases, careful observation was made of the onset of disease symptoms and the course of gestation. Young of animals delivered spontaneously at term were sacrificed at 1 through 28 days of age, as the size of the litter allowed. In addition to the removal of maxillae and mandibles for histologic study, blood samples were inoculated into normal mice in order to test for the presence of LCM virus. Similar procedures of sacrifice and blood testing were done on representative maternal animals in each of the inoculated groups, as well as in the control mothers and young.

The 20 mice inoculated with heat-killed suspensions of LCM virus were followed for the purpose of evaluating the possible influence of the intraperitoneal inoculation of neural tissue per se.

*These animals were made available through the courtesy of Dr. Charles Armstrong.

FINDINGS

The course of pregnancy in mice is significantly affected when there is a concomitant complication by acute lymphocytic choriomeningitis. As noted in Table I, the later in pregnancy that the disease is induced, the greater is the

TABLE I

INOCULATED (DAYS POST BREEDING)	NUMBER OF ANIMALS	NUMBER DELIVERING AT TERM	NUMBER SHOWING ABORTION OR RESORPTION	NUMBER SHOWING NO EVIDENCE OF PROGRESSION OF PREGNANCY	EVIDENCE OF VIREMIA IN DELIVERED YOUNG		
					NUMBER OF LITTERS TESTED	POSITIVE VIREMIA	NEGATIVE VIREMIA
0-6 days	68	7	19	42	7	7	0
7-13 days	63	30	7	26	26	14	12
14 days' term	22	18	0	4	14	7	7

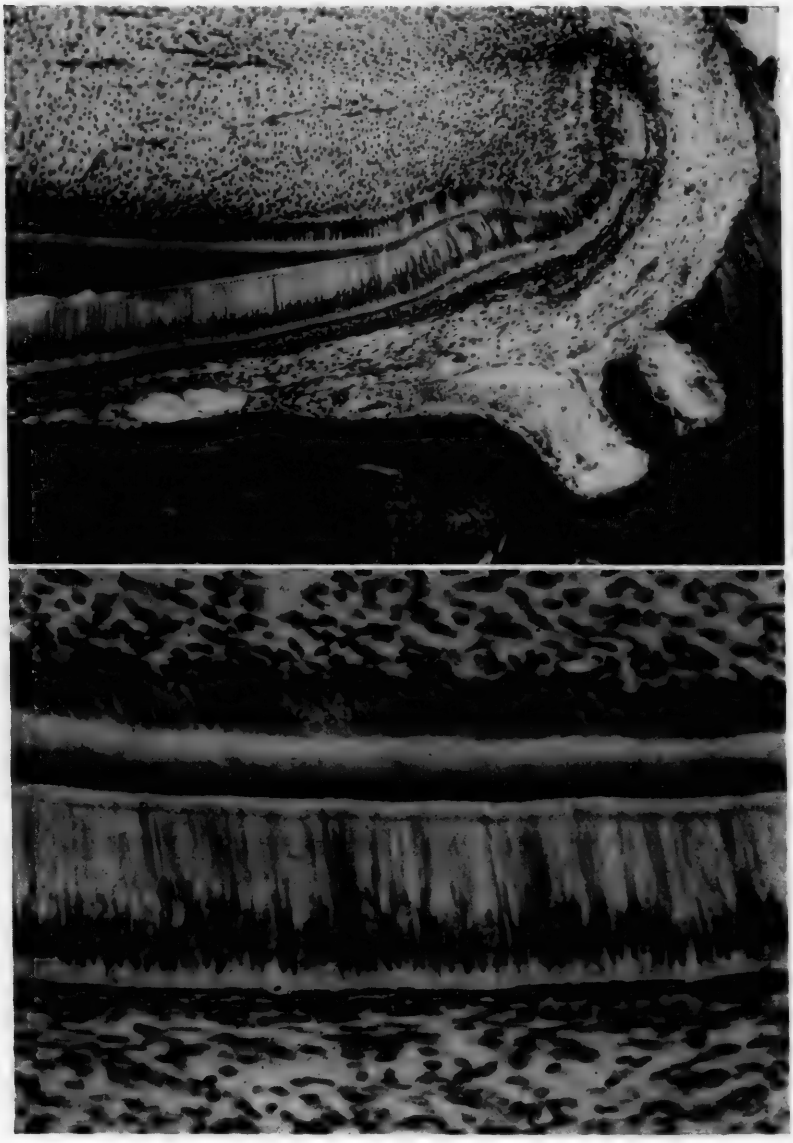
likelihood for successful delivery. Most striking is the occurrence of only 37 term deliveries in a total of 131 bred animals inoculated during the first and second trimesters. Of these delivered litters, 25 (67 per cent) were viable and 12 (33 per cent) nonviable with, however, no significant difference in their average sizes (6.7 and 6.0 young, respectively). Litter size was comparable in inoculated animals, those receiving heat-killed virus, and controls. That the first trimester is undoubtedly the most critical period is evidenced by the term delivery of but 7 of the 68 mice comprising this group. Of the 61 animals that failed to complete a normal course of pregnancy, definite evidence of abortion or resorption was manifested in 19. Since one would expect a high percentage of successful pregnancies in normal mice following appearance of a vaginal plug,[15] it is reasonable to assume that the vast majority of the remaining 42 animals in the first trimester group likewise had abnormal terminations of pregnancy. In the second trimester group, there was a significantly higher percentage of successful term deliveries with a correspondingly decreased incidence of premature interruptions of gestation, whether by resorption, abortion, or unascertained etiology. Of the 22 mice inoculated during the third trimester, 18 delivered viable litters at term; their average size being 6.3 young. The absence of abortion-resorption in this group is most likely explainable either on the basis of little time remaining during pregnancy for such an event to occur or the greater resistance of the gestational organism at this stage. Lending support to the former hypothesis is the probability that the incubation period of the disease following third trimester inoculation would in most instances extend beyond the day of delivery. Consequently, little significance can be attached to findings in this experimental group.

It is of interest to note that of the 43 viable term deliveries, 10 litters died within 4 days of birth and 6 within 10 days. No apparent correlation was noted between these instances of mortality and the time of maternal inoculation. There was some evidence, however, that the litters died of inanition.

With but one exception, all full-term delivered young, regardless of trimester of inoculation or whether viable or stillborn, appeared grossly normal. One

mouse, inoculated on the eleventh day of pregnancy, delivered 2 abnormal young characterized by a rather marked defect of the cranium with extrophy of the brain. A third littermate showed no abnormalities.

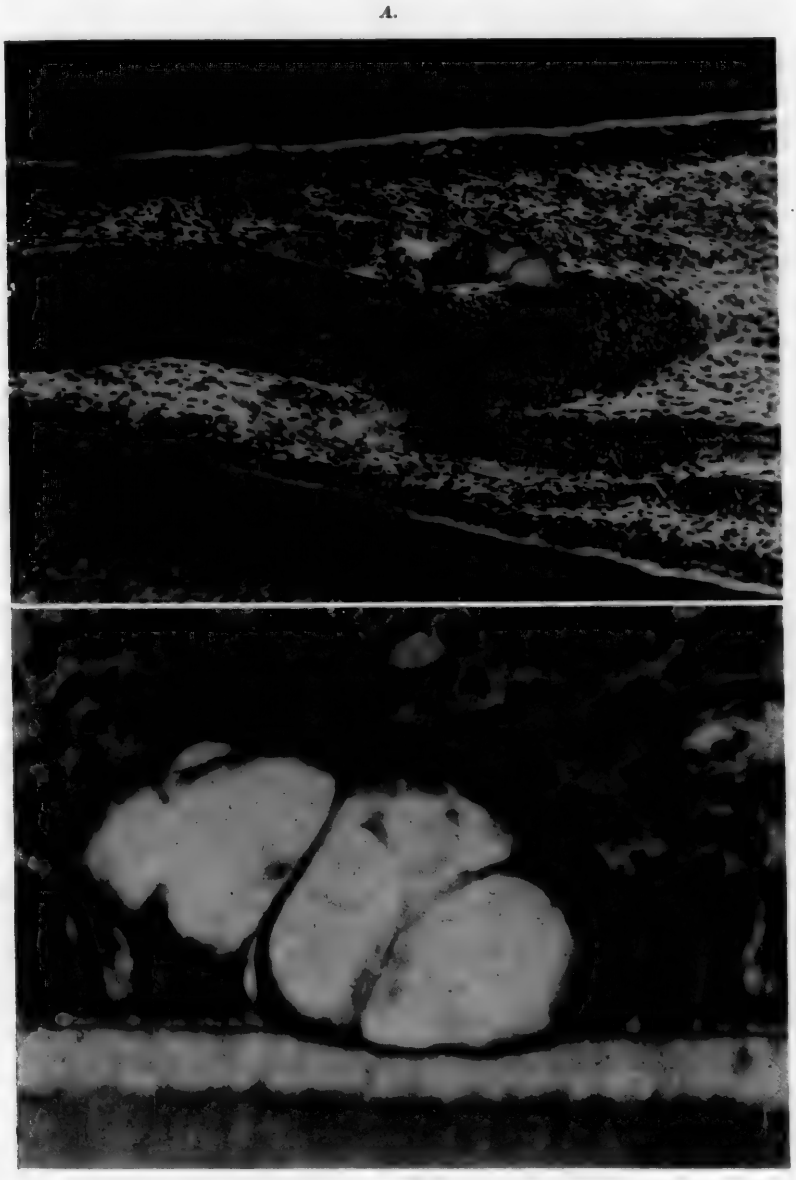
A.



B.

Fig. 1.—*A*, Incisor of control adult mouse showing normal appearance of ameloblasts, enamel matrix, dentin, and pulp. *B*, Incisor from a 1-day-old control offspring of a non-inoculated mother showing tall, regularly arranged ameloblasts underlying a thin strip of early enamel formation. Dentin, predentin, and odontoblastic layer are likewise of normal appearance.

Lending significance to the findings of abnormal pregnancy in the 153 experimental mice was the uniformly successful course of pregnancy terminating in delivery of viable litters (93 per cent) in 30 breeding controls following

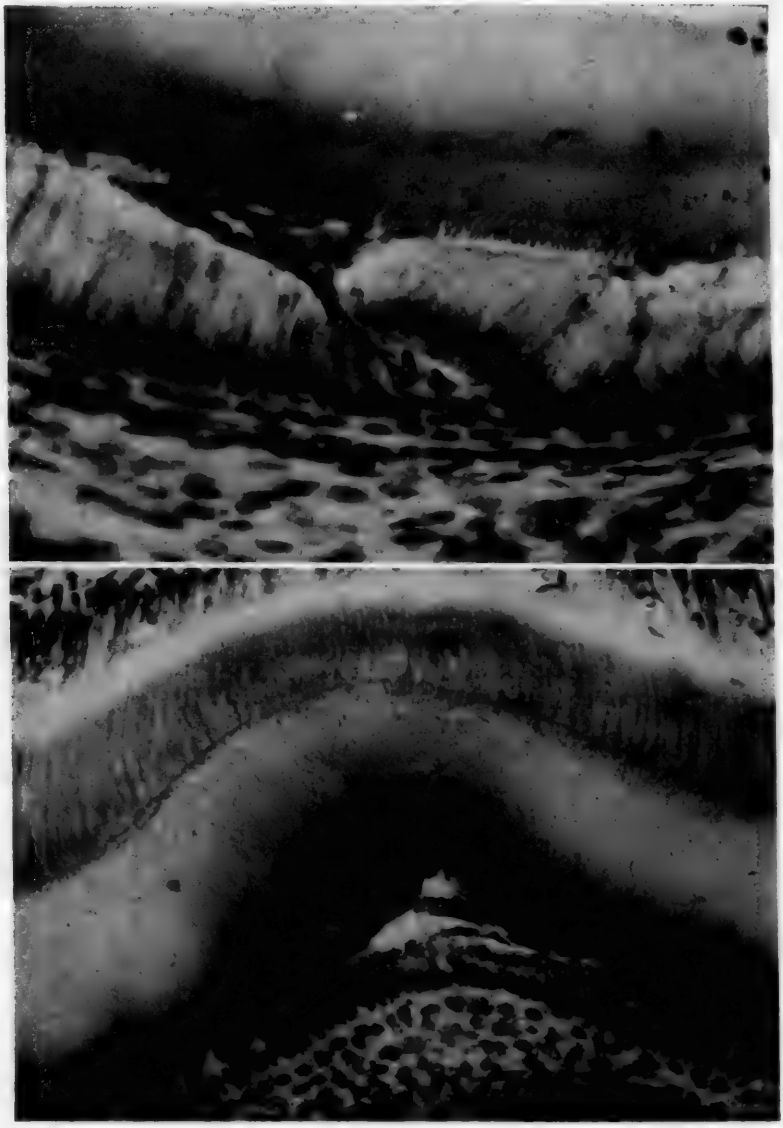


A.

B.

Fig. 2.—*A*, Marked pulpal hyperemia and hemorrhage in a mandibular incisor from a mouse that died of lymphocytic choriomeningitis 8 days post inoculation. *B*, Odontoblastic vacuolization in a maxillary incisor of a mouse sacrificed 17 days after intraperitoneal inoculation of virus.

A.

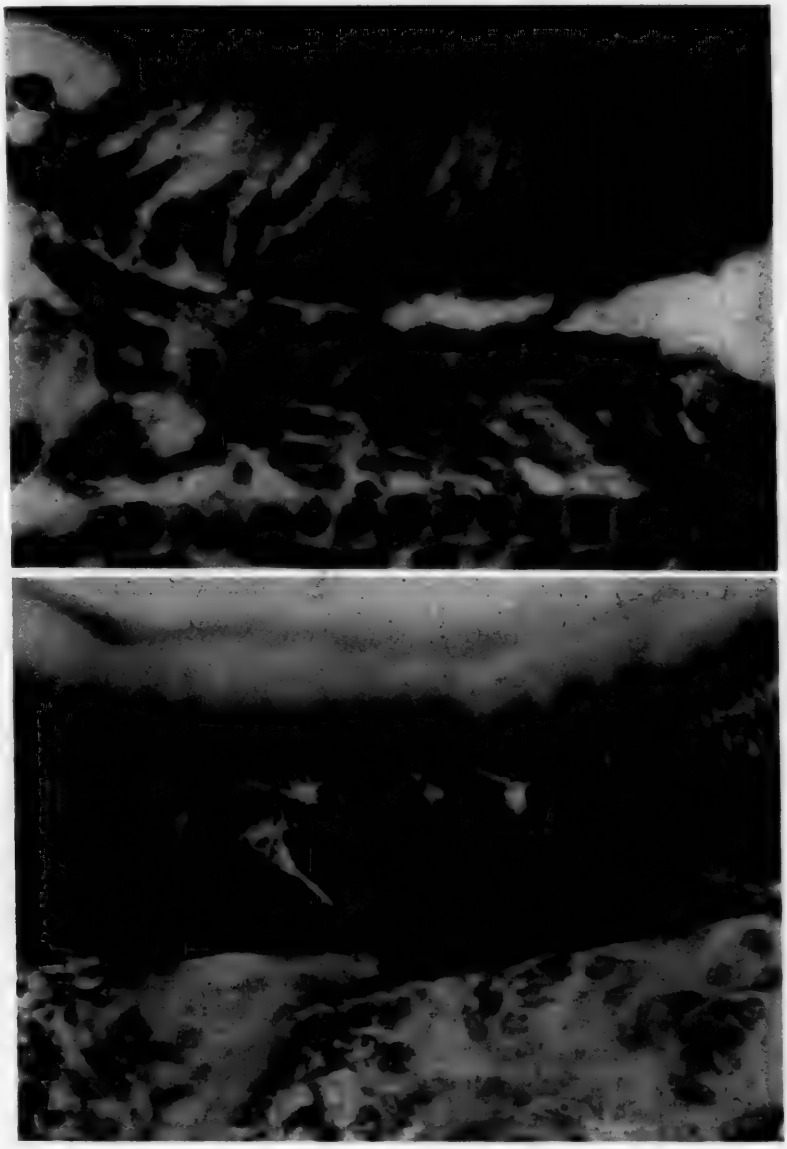


B.

Fig. 3.—*A*, Mandibular molar of a 7-day-old congenitally infected mouse. Ameloblastic layer shows a rather striking discontinuity characterized by an invaginated zone of degenerated cells.

B, Molar from a 7-day-old offspring of a mouse inoculated on the sixth day of gestation. The well-defined, intact layer of ameloblasts is separated from the overlying organic enamel matrix by a degenerated layer of cells and irregular, basophilic staining deposits of enamel-like material.

C.



D.

Fig. 3, *C* and *D*.—*C*, Maxillary incisor of a 7-day-old congenitally infected mouse. A normal-appearing layer of tall columnar ameloblasts is subjacent to the enamel matrix. Separating these cells from the intact stratum intermedium is another layer of cells resembling ameloblasts but characterized by degenerative changes and marked irregularity.

D, Molar from a 7-day-old offspring of a congenitally infected mouse. A number of irregular masses of enamel-like material are present between the enamel surface and ameloblastic layer, causing some compression of cells.

initial diagnosis by vaginal plug formation. This indicates the probability that the vast majority of those experimental animals showing no evidence of progression beyond the appearance of the plug had an interruption of gestation rather than a failure of conception. In those instances of unquestionable abortion or fetal resorption, the probable etiologic role of the disease is quite apparent. That the viable virus causing the acute disease is the complicating factor rather than the macerated neural tissue of the inoculum is evidenced by the normal course of pregnancy in 80 per cent of 20 mice inoculated with heat-killed preparations at varying stages of gestation.

Inasmuch as the LCM virus was selected for study on the basis of its demonstrated ability to cross the placental barrier,[12, 13] the produced congenital infection would necessarily warrant consideration, should any dental abnormalities be found in the young. It is of interest to note that maternal viremia may be detected as early as 48 hours after intraperitoneal inoculation and vary considerably in duration. Although differences in the titers of prepared inocula may, in part, explain the varying duration of viremia, many animals inoculated with equivalent doses from the same suspension source of virus show a like unpredictable course of viremia.

Since there were only 55 deliveries at term in the entire series of 153 mice inoculated with lymphocytic choriomeningitis virus, the number of young available for testing of transplacental infection was appreciably limited. Also pertinent to a proper evaluation of such findings is that these litters were not proportionally distributed in relation to the trimester of maternal inoculation. As indicated previously, this can be attributed to the greater vulnerability of early pregnancy to lymphocytic choriomeningitis. It seems significant, nevertheless, that of 7 litters delivered of mothers inoculated during their first trimester of pregnancy, all gave positive evidence of viremia, whereas, of 26 litters tested in the second trimester inoculated group, only 14 (54 per cent) showed evidence of viremia. In 14 tested litters of mothers inoculated during their last week of pregnancy, 7 (50 per cent) proved to have LCM virus in their circulating blood. The ages of the young at time of testing, which ranged from 1 to 28 days, had no apparent relation to the presence or absence of a viremia.

Histologic examination of serially sectioned teeth and supporting structures of control maternal animals and their offspring (Fig. 1) shows a typically normal picture characterized in the incisors by an intact and uniformly arranged layer of ameloblasts which overlies a regularly formed enamel matrix. Dentin, predentin, and pulpal tissue, including the odontoblastic zone, are also of normal appearance. The fully developed posterior teeth of parent animals, as well as the developing molars of their young, show like evidence of normal structure.

Little variation from the control series is noted in the incisor and molar teeth of inoculated maternal mice. The only consistent departure from normal is pulpal hyperemia and hemorrhage (Fig. 2, *A*). Odontoblastic vacuolization is present in fewer cases (Fig. 2, *B*). In contrast to these observations is the relatively infrequent occurrence of dental abnormalities in the offspring of infected mothers. In most instances these were young that showed the presence

of virus in their circulating blood. When present, the abnormalities are noted predominantly in those young sacrificed at 7 days of age and are characterized chiefly by disturbances in amelogenesis. The ameloblasts in these cases often present a folded or double-layer appearance suggesting artefact (Fig. 3). However, they are considered to be true pathologic changes on the basis of their consistent presence in multiple, serial sections and their probable mechanism of formation which may be attributed either to a disturbance in cell alignment as illustrated in Fig. 3, *A*, or a degeneration of cells beneath a normal-appearing ameloblastic layer as suggested in Fig. 3, *B* and *C*. Other of the abnormal young show irregular, enamellike deposits overlying the organic enamel matrix (Fig. 3, *D*). The subjacent inner enamel epithelium is accordingly compressed in this region although not otherwise abnormal in appearance. It is probable that the cellular change responsible for the hyperplastic enamel formation was a reversible one. Suggesting a more previous and transitory disturbance in amelogenesis is the presence of a prominent incremental line as illustrated in Fig. 4.

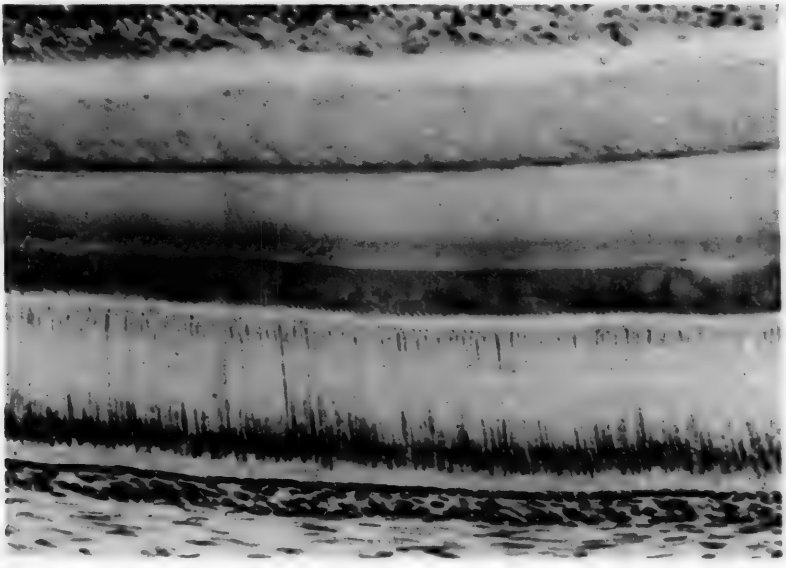


Fig. 4.—Mandibular incisor of a 7-day-old congenitally infected mouse showing prominent incremental line in the enamel. A parallel but appreciably wider band of acidophilic-staining matrix characterizes the more recently formed enamel. Ameloblasts are of normal appearance as are the dentin, predentin, and pulp.

DISCUSSION

It has been shown that lymphocytic choriomeningitis appreciably affects the course of pregnancy in mice and that such effect is particularly evident when the disease is induced during the earlier stages of gestation. Although this influence is manifested by abortion or resorption in but 26 cases (Table I), it is suggested that the great majority of the 72 remaining cases of unsuccessful

pregnancy had like terminations which, however, escaped observation. It is certainly most unlikely that so large a number of mice giving evidence of vaginal plug formation after copulation would have failed to conceive. This contention is supported by the observations of Parkes[15] who found that 71 out of 88 mice became pregnant following the appearance of a vaginal plug. An even higher percentage was found in the present study with 28 out of 30 breeding control animals showing a successful course of pregnancy after vaginal plug formation.

Although the studies of Traub[12] and Haas[13] included mice inoculated shortly before and during pregnancy, the only mention of untoward influences on gestation was several instances of stillbirths, with no abortions being observed. It is of interest in this regard to note the evidence in humans indicating the critical nature of first trimester pregnancy. Analyzing a series of 591 cases of spontaneous abortion that occurred during the first 6 months of pregnancy, Simons[16] found 516 occurring prior to the third month.

The rather marked variability in duration of viremia in mature mice, ranging from 48 hours to 76 days post inoculation, is in striking contrast to the extremely long periods of viremia that prevail in congenitally infected animals.[17] Considering that relatively few of the mature mice gave evidence of circulating virus beyond the fortieth day, these findings are in general agreement with those of Haas[13] who reported a marked difference in the nature of infection when established in utero or after maturity.

Contrary to the consistent development of lymphocytic choriomeningitis infection in inoculated maternal mice, it has been demonstrated that their offspring show an increasing evidence of negative testing for virus as the period of maternal inoculation is delayed beyond the first trimester of pregnancy. This may indicate an increasing difficulty of placental passage of LCM virus associated with advancing gestation. Whether a similar explanation of enhanced placental permeability during first trimester pregnancy may, in part, apply to the critical nature of this period relative to other maternal virus diseases, such as rubella, is conjectural.

Particular attention was directed toward the possibility of lymphocytic choriomeningitis affecting maternal and fetal dental tissues. This eventuality was not considered too unlikely despite the essentially neurotropic nature of the virus. Such changes as polyserositis, fatty degeneration of liver and kidneys, and focal necrosis, thrombosis and lymphocytic infiltrations in a variety of tissues and organ sites were observed by Lillie and Armstrong[14] in mice inoculated by the intraperitoneal route. Consequently, the rather consistent finding of marked pulpal hyperemia and interstitial hemorrhage in incisor and molar teeth may be correlated with the almost uniform induction of lymphocytic choriomeningitis in inoculated adult mice, and thereby provide some evidence of cause and effect. On the other hand, the relatively infrequent finding of dental abnormalities in the young of mice inoculated during pregnancy does not afford sufficient evidence for a consistent virus etiology. It is of some significance in this regard, that the changes in congenitally infected animals are

found predominantly in the enamel organ and that no pulpal pathosis, analogous to that found in parent mice, is noted. Furthermore, the presence of demonstrable congenital infection does not insure the occurrence of an abnormal amelogenesis. It would appear, therefore, that either the placental passage of LCM virus is not a consistent or direct contributing factor to congenital dental changes, or that fetal tissues show a marked variation in susceptibility to injurious agents, whether they be the virus in question or agents unknown.

Despite the fact that placental permeability to LCM virus seems to decrease as pregnancy progresses, and that such evidence would support the contention of many that first trimester maternal disease is most apt to cause fetal damage, one cannot underestimate the likelihood of other untoward influences incident to the primary disease that may be operative during pregnancy.

SUMMARY AND CONCLUSIONS

The present study was designed to further explore the question of prenatal influences on tooth development with particular reference to the rate of transplacental virus passage as an etiologic factor.

Inasmuch as available evidence regarding the relationship of such virus diseases as rubella to congenital abnormalities is based almost entirely upon clinical observation, it seemed advisable to study other viral infections of known pathogenicity for common laboratory animals. Since relatively few of these meet the required criterion of ability to cross the placenta, any virus so selected might possibly have some limitations. Although the virus of lymphocytic choriomeningitis is essentially neurotropic, previous studies have shown direct influences upon other elemental tissues and organ sites.

On the basis of studies on 203 female Swiss mice and their offspring, the following observations were made:

1. The course of pregnancy is adversely affected when there is a concomitant complication by acute lymphocytic choriomeningitis.

2. The relatively high percentage of abortions, resorptions, and other instances of nonprogression of pregnancy are most frequent when the maternal disease is induced during the early stages of gestation.

3. The reliability of vaginal plug formation as a criterion for the diagnosis of conception in the 153 mice comprising the virus-inoculated group is indicated by the number of successful terminations of pregnancy (93 per cent) in a control series of animals.

4. The duration of viremia in adult mice following LCM virus inoculation is rather brief.

5. The demonstration of fewer instances of congenital infection in the offspring of mothers inoculated during the later stages of pregnancy suggests a correlation between placental permeability to LCM virus and the period of gestation when the maternal disease is induced.

6. Pulpal hyperemia and hemorrhage are consistent findings in the incisor and molar teeth of mice following inoculation with the virus of lymphocytic choriomeningitis. Odontoblastic degeneration is noted occasionally.

7. Dental abnormalities are relatively infrequent in the young of infected mothers. However, when present, they are characterized by disturbed amelogenesis rather than by pulpal changes as observed in inoculated maternal mice.

8. Congenital dental abnormalities occur predominantly in young that demonstrate a viremia. However, similar changes are noted in mice that fail to give evidence of congenital infection.

9. The possibility of an abnormal maternal metabolism incident to the lymphocytic choriomeningitis must also be considered of etiologic importance in congenital dental abnormalities.

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TOOTH DEVELOPMENT AND ERUPTION

II. A Biometric Analysis of the Histologic Relationship of the Root of the Mandibular First Molar of the Rat in Growth and Eruption to the Lower Border of the Mandible

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INTRODUCTION

CLINICALLY and histologically it is quite apparent that under normal circumstances a tooth develops and erupts from within the bone proper to a relatively definite position in the outside environment of the oral cavity. How this migration is made possible has yet to be answered. Any satisfactory explanation of such a complex biologic process needs the correlation of data from both the clinical and basic fields. The mechanisms by which eruption is initiated or enhanced have been explored but a description of the process eruption in itself has been neglected. It is hoped that this and related studies will help contribute to an understanding of the eruption process from these aspects.

Since a basis for measurement appears to be a prime requisite in tooth eruption, it was believed one could be evolved from certain measurements obtained from serial sections of variously aged rats from birth upward. Although this would not be a true indication of one growth process, these measurements could be viewed from a comparative growth, or growth-ratio standpoint, and thus be a valuable addition to our study.

Such a study was undertaken, and this paper presents the materials, methods, and results obtained by the biometric analysis of how the root of the first mandibular molar in the rat is maintained spatially in the growing mandible during the growth and eruption period.

MATERIALS AND METHODS

Materials.—Ten rats of thoroughbred Norwegian strain ranging from newborn to 31 days of age were utilized. The animals were fed a normal diet of fox chow and oats (fresh vegetables once weekly) and caged in the usual fashion.

Preparation of tissues was carried out in the following manner: The rats were anesthetized and immediately sacrificed by decapitation. The jaws were separated and fixed in 10 per cent neutral Formol for periods from 24 hours to 5 days. The jaws of the older animals were skinned prior to fixation to permit better penetration of the fixing solution. They were washed for 24

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TABLE I
MEASUREMENTS BY MEANS OF DIVIDERS AND METRIC SCALE

AGE IN DAYS	AB	AC	CB	FB	FC	EB	EC	OB OR OA	OC	OD	OE	ANGLE OF ERUPTION
					Measurements in millimeters							
1	45.0	20.8	25.4	43.0	19.7			22.5	4.8	12.0		50°
6	65.0	30.6	36.7	57.8	24.3			32.7	8.0	12.0		42°
9	79.4	37.6	45.3	73.4	31.6	54.3	8.7	39.5	10.8	16.2	16.3	46°
12	86.4	46.6	43.7	86.8	47.3	60.2	17.4	43.8	10.0	16.2	17.0	57°
15	87.4	39.5	48.6	89.0	40.4	71.0	21.9	44.5	7.9	17.0	26.6	64°
18	84.0	37.5	50.2	87.0	43.7	71.3	26.2	42.2	9.5	16.6	29.6	48°
24	95.0	43.3	53.1	108.7	59.8	87.7	36.8	47.6	11.0	16.0	39.5	55°
26	99.5	47.3	56.8	110.8	61.0	90.0	38.6	50.0	12.3	19.0	39.9	53°
29	87.8	35.8	56.4	110.3	54.0	84.1	32.8	44.0	13.0	15.7	39.4	50°
31	92.0	37.0	58.8	99.5	49.5	87.6	34.0	46.5	14.0	17.0	40.7	50°

A = Tip of buccal alveolar crest.
B = Lower border of mandible directly below incisor.
C = Apex of root or base of dental papilla at the point of entrance of the vessels.
D = A buccal point of mandibular width on the horizontal plane.
E = Cervical line (buccal).
F = Tip of buccal cusp.
O = Geometric center of expansion.

hours and then dehydrated through successive changes of alcohol (70 per cent through 100 per cent and then alcohol and ether). The tissues were kept 24 hours in each change of alcohol. Decalcification was carried out during the 80 per cent alcohol stage in a mixture of 5 parts of concentrated nitric acid to 100 parts of 80 per cent alcohol. Decalcification required periods up to 2 weeks.* The tissues were then infiltrated (with 10 per cent and 25 per cent celloidin) for approximately 1-month periods and embedded in 25 per cent celloidin.

Each one-half mandible was cut serially at from 6 to 8 microns in a sliding microtone with the tooth in buccolingual aspect. Each specimen was oriented in such a manner as to attempt duplication of plane of cut. Every eleventh section was stained with hematoxylin and eosin as a check on orientation. Approximately 40 sections encompassing the midsection of the developing tooth were then stained using the Masson-Mallory connective tissue, iron hematoxylin aniline blue and van Gieson stains and mounted in Euparal.

Methods.—Microscopic studies of these sections were made, and entrance of the blood vessels at the developing apical foramen area was used as the criterion for the middle section. The middle section was photographed at a magnification of 25 times. One plane that would be common to all sections was drawn on each print from the point which could be considered the tip of the buccal alveolar crest to a point on the outer aspect of the lower border of the mandible directly beneath the incisor. The points were respectively labeled *A* and *B*. This line of given distance for each specimen was then bisected to produce another plane *GD* which then also would be common to all sections. As a means of arbitrarily placing a point which would be common to each specimen as well as to the group, a geometric center for each specimen was determined by bisecting the line *GD*, and the point of intersection was labeled point *O* (Fig. 1).

The measurements by means of dividers and metric scale seen in Table I were recorded. Points *A* and *B* have been identified above. Point *C* denotes the point at the base of the dental papilla or the apical area of the developing root at which the entrance of the blood vessels occurs. Point *D* is that point on the perpendicular plane to *AB* on which the outer or buccal aspect of the mandibular bone falls. Point *G* represents the inner or lingual aspect of the mandibular bone on that plane. Point *E* is the cervical line (cemento-enamel junction) on the buccal aspect of the tooth. Point *F* is the tip of the buccal cusp of the tooth.

From the measurements recorded it was desired to obtain a graphic picture which would show the relationship of the growth rates of the various structural distances in view of the fact that one specimen could not be chronologically followed on a histologic basis. Figs. 2 and 3 were thus constructed. The charts represent the patterns obtained for each rat.

*Sectioning was difficult in the older-aged animals starting with the 26-day rat. Decalcification should be continued to one month, and the usual tests for calcium not considered to be reliable.

Because each rat was of a different age, each measurement for one rat had to be related to its respective measurement in another age by a proportionate or relative value. Since point *O* was developed as the only common point of reference for each animal, this point was used as a basis for measurement, and the landmarks *A* and *B* were plotted with respect to this point. Then the points *C*, *F*, and *E* were plotted in relationship to their respective points *B* (Fig. 4). In this manner the behavior of the pertinent landmarks could be followed in relationship to the lower border of the mandible.

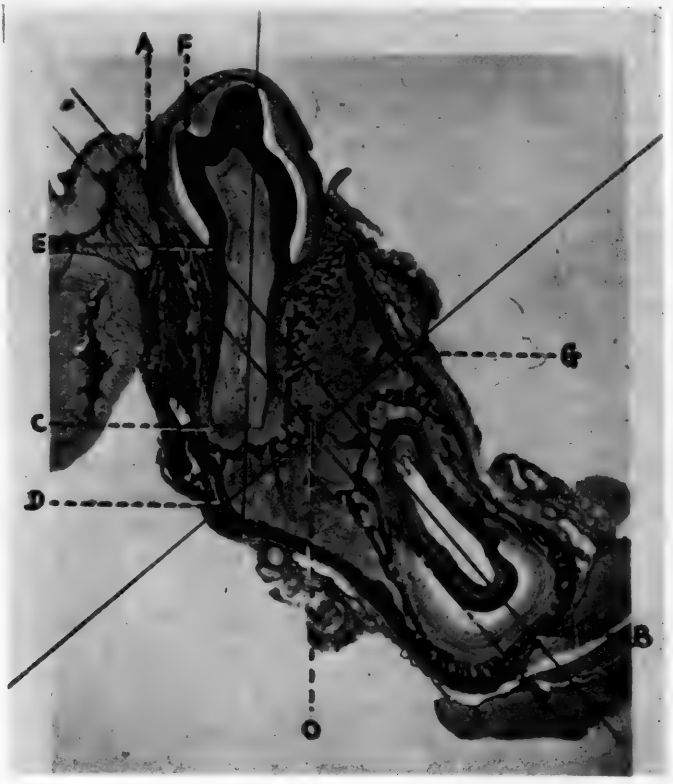

Fig. 1.—Method of determining measurements from histologic specimens (18-day rat).

In order to position point *C* in relationship to both *A* and *B*, the points *A*, *B*, and *C* were plotted in relationship to their respective point *O*. The points *C* were plotted from their respective points *A* and points *B* (Fig. 5). Thus the behavior of points *A*, *B*, and *C* with respect to the geometric center and themselves could be followed.

The point *C* was then assumed to be a fixed point, and points *F*, *A*, *E*, and *B* were plotted to determine the relationship of the apex to these points under such an assumption (Fig. 6).

Since the angle of eruption of the tooth with respect to the buccolingual plane (*GD*) had been measured, a plot of this was made (Fig. 7) to determine the movements of the tooth in the histologic preparations during its eruption.

It was felt that although all the above plots were essential to a clear picture of the relationship of the different selected histologic landmarks to each other and to their respective landmarks on the other specimens, they did not accurately establish a proportion or ratio study which now appeared to be the necessary step in describing how variable points in variable specimens could be spatially related to each other. Hence the ratios of growth between certain selected distances (Table II) were plotted against time (Fig. 8). By the simple arithmetical procedure of subtraction of such ratios to produce a constant—or by the graphic similarity of curves—the points or distances desired would thus be effectively portrayed as behaving in a specific manner during a specific time period regardless of the necessity of utilizing different specimens.

TABLE II
RATIOS OF GROWTH BETWEEN CERTAIN SELECTED DISTANCES PLOTTED AGAINST TIME

	AGE IN DAYS									
	1	6	9	12	15	18	24	26	29	31
	Measurements in millimeters									
CA	20.8	30.6	37.6	46.6	39.5	37.5	43.3	47.3	35.8	37.0
CB	25.4	36.7	45.3	43.7	48.6	50.2	53.1	56.8	56.4	58.8
ratio	.82	.83	.83	1.07	.81	.75	.82	.83	.635	.63
CE			8.7	17.4	21.9	26.2	36.8	38.6	32.8	34.0
CB			45.3	43.7	48.6	50.2	53.1	56.8	56.4	58.8
ratio			.19	.40	.45	.52	.69	.68	.58	.58
CF	19.7	24.3	31.6	47.3	40.4	43.7	59.8	61.0	54.0	49.5
CB	25.4	36.7	45.3	43.7	48.6	50.2	53.1	56.8	56.4	58.8
ratio	.78	.66	.70	1.08	.83	.87	1.13	1.07	.98	.84
BA	45.0	65.0	79.4	86.4	87.4	84.0	95.0	99.5	87.8	92.0
BC	25.4	36.7	45.3	43.7	48.6	50.2	53.1	56.8	56.4	58.8
ratio	1.77	1.77	1.75	1.98	1.80	1.67	1.79	1.75	1.56	1.57
BF	43.0	57.8	73.4	86.8	89.0	87.0	108.7	110.8	110.3	99.5
BC	25.4	36.7	45.3	43.7	48.6	50.2	53.1	56.8	56.4	58.8
ratio	1.73	1.58	1.62	1.99	1.83	1.73	2.02	1.95	1.96	1.69
BE			54.3	60.2	71.0	71.3	87.7	90.0	84.1	87.6
BC			45.3	43.7	48.6	50.2	53.1	56.8	56.4	58.8
ratio			1.20	1.38	1.46	1.42	1.65	1.58	1.49	1.49
CO	4.8	8.0	10.8	10.0	7.9	9.5	11.0	12.3	13.0	14.0
CB	25.4	36.7	45.3	43.7	48.6	50.2	53.1	56.8	56.4	58.8
ratio	.19	.22	.24	.24	.16	.19	.21	.22	.23	.24
CO	4.8	8.0	10.8	10.0	7.9	9.5	11.0	12.3	13.0	14.0
CA	20.8	30.6	37.6	46.6	39.5	37.5	43.3	47.3	35.8	37.0
ratio	.23	.26	.29	.21	.20	.25	.25	.26	.36	.38

OBSERVATIONS AND FINDINGS

The columnar plot in Fig. 2 compares the growth distances of all the rats. It shows a striking similarity in pattern, despite a disrupted continuity of the growth process which would not be seen were it possible to follow one animal. All columns, for example, maintain certain positions with respect to

one another except in Rat 12 in which case (although the change is not striking) Columns 3 and 4 have lost the relationship found in all the other animals. The fact that this dissimilarity appears at the initial stages of root formation indicates that with root formation a downgrowth of the root into the jaw occurs prior to the progressive eruption of the tooth. Since the distance from the

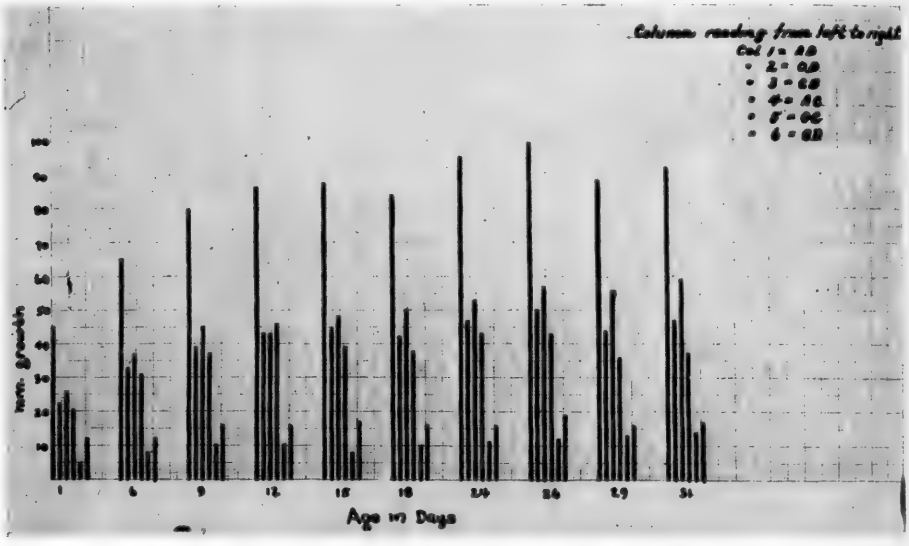

Fig. 2.—A comparison of mandibular growth trends in rats from birth to 31 days.

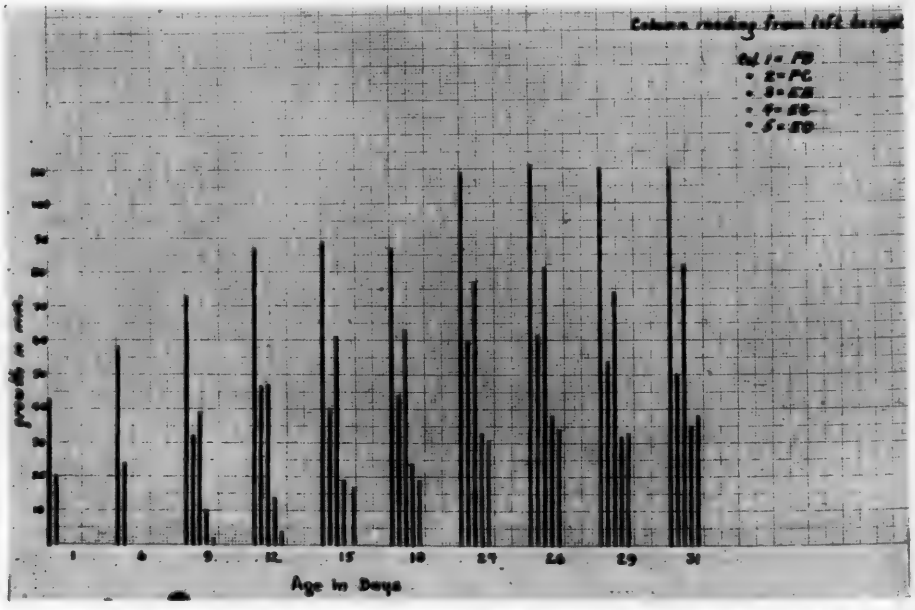

Fig. 3.—A comparison of tooth-mandibular growth trends in rats from birth to 31 days.

tip of the alveolar crest to the apex of the root is greater than that in Rats 9 and 15, and since the tooth distance from the tip of the cusp to the apex has increased, the observation of downgrowth of the root with no advance of the tooth in the occlusal direction at the initial stages of root formation is given support.

A rather interesting finding is the insignificant growth of the mandible in width (Column 6 shows only one-half the mandibular width). At birth, mandibular width is approximately one-half the mandibular height; whereas at 31 days, it more nearly approximates one-third that distance. Mandibular height appears to have reached its maximum with eruption of the tooth into the oral cavity (Column 1); however, this cannot be considered a conclusive bit of evidence until studies including older aged animals have been made. At least the greatest rate of growth occurs within the first 12 days (again the time of root formation) at which time the height has been nearly doubled. After 12 days, growth of the mandible in height is relatively slight.

Similarity of position-relationship of columns in Fig. 3 exists in Columns 1, 2, 3, and 4 with the exception again of Rat 12. The increase in tooth length despite the stability of the cervical line to lower border distance progression, again indicates a possible downgrowth of the root at the initial stages of root formation (since tooth length is the result of root length at 12 days). Columns 4 and 5 show that the distance from the cervical line to the apex gradually decreases in proportion to the distance from the cervical line to the geometric center until at 29 days the latter distance is the greater. This indicates that the root apex gradually moves toward the occlusal. Column 4 indicates that root growth occurs at a steady rate until root completion, which is approximately 26 days, or at a time just after eruption of the tooth into the oral cavity. Columns 1 and 2 have not been seriously considered, because of the liability of error when measurement is taken from the tip of the cusp. The crown contour and the considerable deviation of the cusp tip when but a slight change in angulation occurs necessitates this omission.

From Fig. 4 it may be seen that with respect to the lower border of the mandible, the tip of the cusp appears to maintain a uniform distance from that border (with a slight decrease reaching its maximum in the 6-day rat) until the 15-day rat, at which time the tip of the cusp rapidly moves away to reach its greatest distance from the lower border in the 29-day rat. The cervical line maintains a steady increase in distance from the lower border during this 31-day period with the most rapid rise occurring between the 12-day rat and the 24-day rat—the days of most active root formation. The apex of the root over the entire 31-day period very slowly increases its distance from the lower border of the mandible. In the 12-day rat, which depicts the stage of initial root formation, the apex takes its only deviation toward the lower border. This again demonstrates downgrowth of the root prior to eruption of the tooth in an occlusal direction. The most active movement away from the lower border occurs between the 12-day rat and 18-day rat. The period just prior to eruption of the crown into the oral cavity (18 to 24 days) demonstrates a slowing down of the occlusal movement.

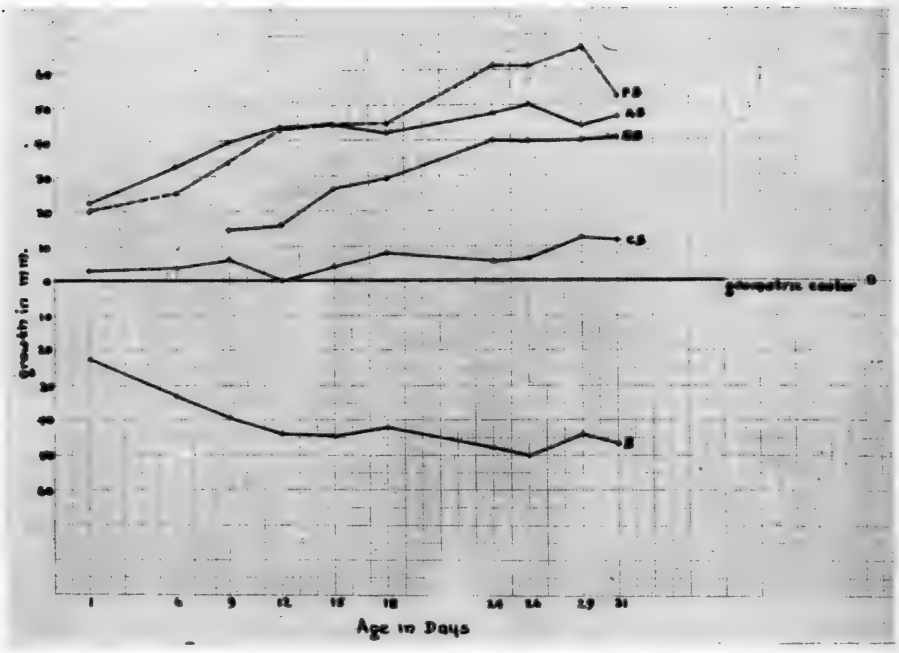

Fig. 4.—Relationship of molar to lower border of mandible in rats from birth to 31 days.

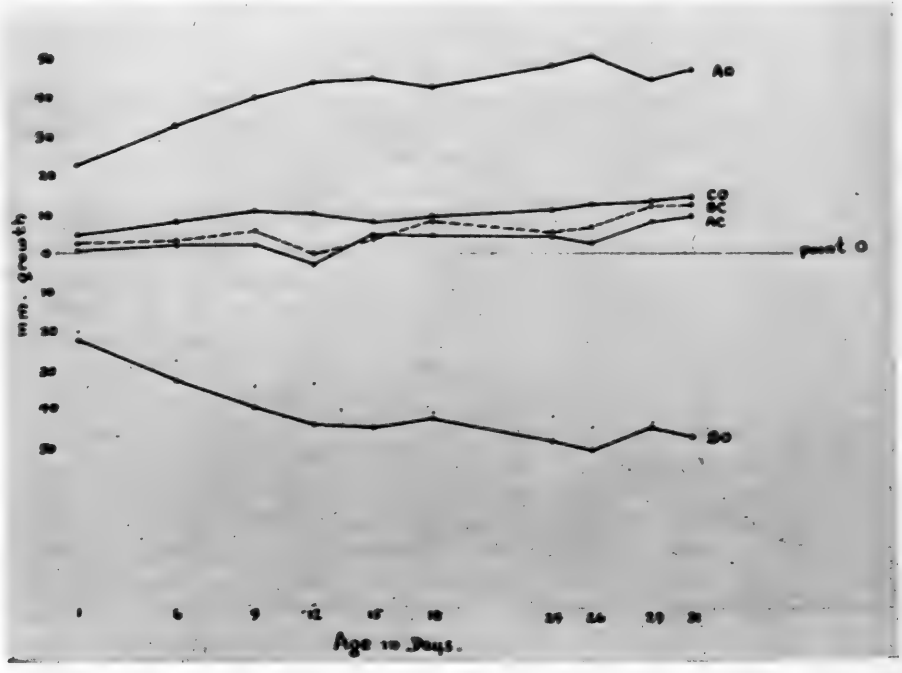

Fig. 5.—Relationship of apex of the root of the first mandibular molar to the buccal alveolar crest and lower border of the mandible in rats from birth to 31 days.

The apex of the root when plotted with respect to both the lower border of the mandible and the crest of the alveolar ridge on the same chart gives a rather clear picture of its position during the growth and development period studied (Fig. 5). Again, the apex is seen to move away from the lower border or toward the alveolar crest at a very slow rate. Until the twelfth day (the stage of initial root formation) only an insignificant movement is noted; however, the apex, in the 12-day rat, is seen to be positioned nearer the lower border

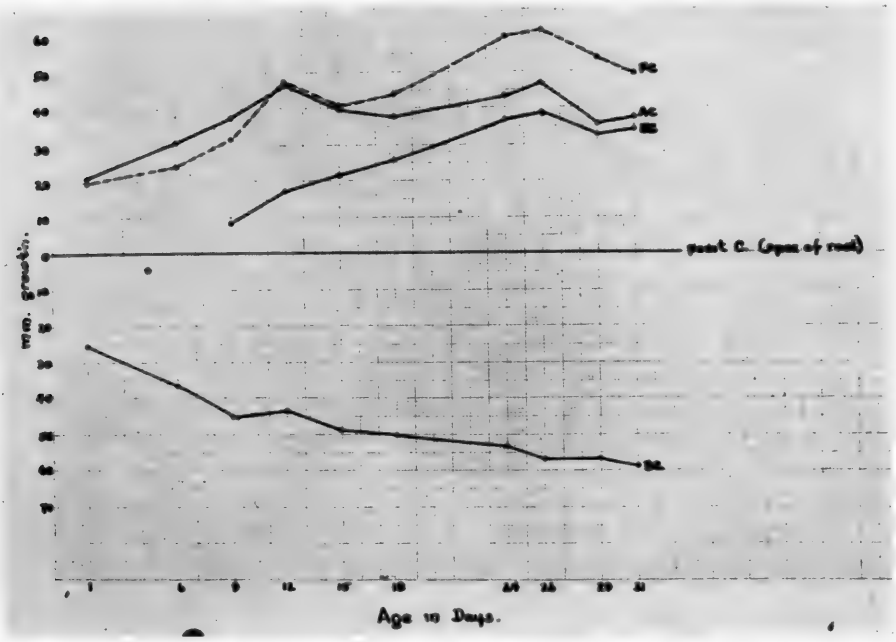

Fig. 6.—Relationship of the lower border and alveolar crest of the mandible and two coronal points of the first molar to the apex of the first molar root when a fixed position for the apex is assumed in rats from birth to 31 days.

than at any time, and indicates a slight downward growth of the apex into the jaw. A rapid movement away from the lower border immediately follows the initial phase of root formation to level off at the 18-day rat, and then slowly move away again after root formation is complete in the 26-day rat. The plot *CO* was drawn merely to indicate that the apex was not identical with the geometric center but also steadily increased its distance from it during the course of root formation and eruption.

Assuming the apex of the root a fixed point in mandibular growth, we find that Fig. 6 shows the growth and development of the tooth itself in detail. Root formation appears to have reached completion at the 26-day rat, and full eruption ceases soon thereafter or at the 29-day rat. The buccal cervical line remains constant to the buccal alveolar crest. The abrupt change in curve *FC* at the 12-day rat indicates the crown portion of the tooth was sectioned through a greater occluso-gingival dimension, for the curve *EC* does not take

the same change. Were one to consider the lower border in relationship to this point, one would have to assume the lower border moved steadily away from the apex at a much faster rate than the alveolar crest approached the apex, or in words we better understand, that the apex moves rapidly and continually away from the lower border. In this case the relationship is apparent only between the two points B and C with respect to no other point.

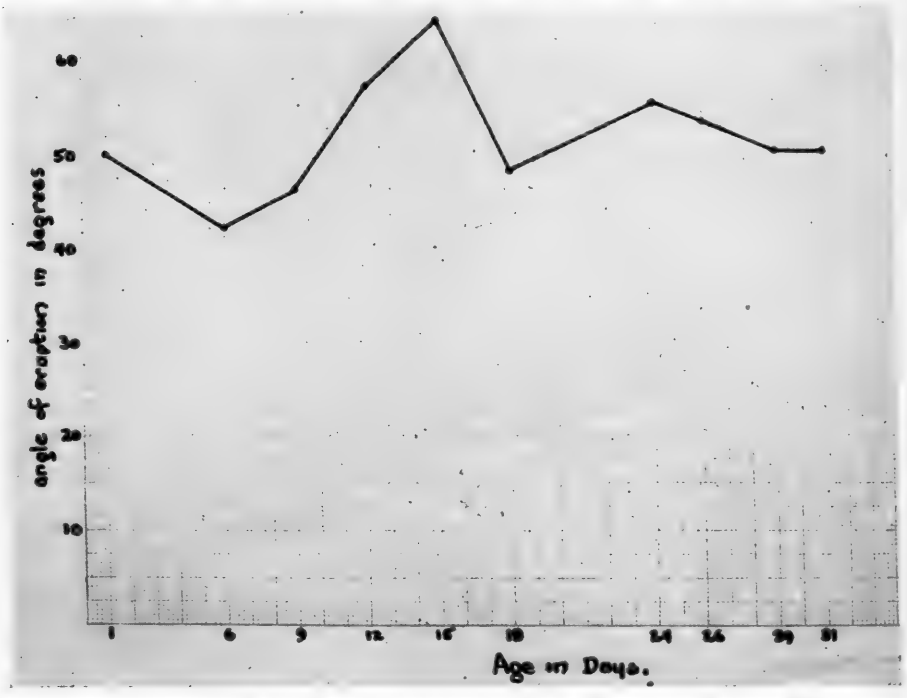

Fig. 7.—Angle of eruption of the first mandibular molar to a buccolingual plane in rats from birth to 31 days.

The angle of eruption (Fig. 7) is variable during the period Rat 6 to Rat 15 (that period just preceding root formation up to and including the period following the initial stages of root formation), but becomes relatively stable from the time just prior to eruption of the crown into the oral cavity until the time last studied.

The curves obtained by plotting growth-ratio figures (Fig. 8) established a similarity seen in curves $\frac{BA}{BC}$ and $\frac{CA}{BC}$ and also $\frac{BE}{BC}$ and $\frac{CE}{BC}$ which by interpretation leads one to assume that the apex of the root maintains a discrete position during growth and eruption with regard to the outer magnitudes of the growing mandible. Curve $\frac{AB}{BC}$ indicates that the apex gradually moves away from the lower border with respect to the increasing over-all height of the mandible. The fact that with respect to the distance AC the apex also moves gradually away from the lower border but produces a relatively identical

curve and thus a constant to the former plot, indicates that the apex, although moving away from the lower border, maintains a constant relationship with the over-all growth pattern. Curves $\frac{BE}{BC}$ and $\frac{CE}{CB}$ show that the distance from the apex to the lower border increases at a constant ratio with respect

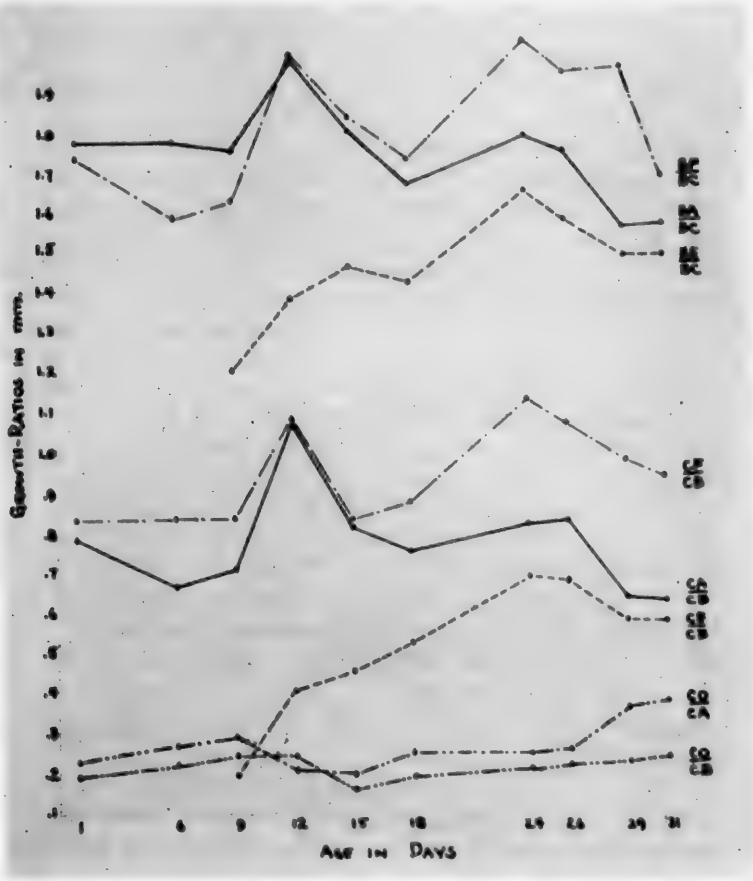

Fig. 8.—Growth-ratios of selected distances in rats from birth to 31 days.

to the distances from the cervical line to the lower border and cervical line to the apex; hence the same interpretation can be derived—that the apex maintains a constant relationship to the lower border in the over-all growth and eruption picture yet does move away from it with respect to that point alone.

Curves $\frac{BF}{BC}$ and $\frac{CF}{BC}$ to a limited extent allow this same interpretation.

Curves $\frac{CO}{CA}$ and $\frac{CO}{CB}$ confirm to a limited degree point O as being a geometric center about which the apex of the root maintains a close relationship until root formation is completed and active eruption has ceased (24 to 26 days).

DISCUSSION

The purpose of this study has not been to refute, formulate, or confirm any hypothesis; rather, it has been to establish as accurately as possible the apex of the growing root spatially in the growing mandible.

It is realized that a study of 10 rats is far from a thorough and complete one. A great many more of the younger and older aged specimens must be studied, especially about the 12-day period. The weight and length of each animal should have been recorded prior to its sacrifice. A more accurate means of orienting the specimen in the celloidin block and establishing an exact duplication in plane of cut would be invaluable.

The mode and reasons for the establishment of a geometric center might well be questioned. Brodie stated, "The superposing of various points as centers is nothing more than a temporary assumption that may have no basis in fact. It can be stated with certainty only that one part behaves in such and such a manner in relation to some other part; i.e., that the distance between certain points increases in a certain direction and at a certain rate."

The developing tooth affords a perplexing problem when the superpositioning of anatomical points is used. Hence the attempt at considering the mandible during its period of growth and development as a subject suspended in space necessitated the establishment of a point somewhere in the main body which could be the center from the outer magnitudes of each specimen and in that respect be related to that same center in other specimens, and yet not be a fixed point with respect to any anatomic structure.

SUMMARY AND CONCLUSIONS

A biometric study to determine how the root of the mandibular first molar in the rat is maintained spatially in the growing mandible during the growth and eruption period in relationship to the lower border of the mandible has produced the following interpretations:

a. During the period of growth of the mandible and growth and eruption of the first molar, the apex of the root of the tooth maintains a constant relationship to the lower border and alveolar crest of the mandible, but gradually moves away from the lower border itself.

b. Growth of the mandible in width, when computed from buccolingual sections made through the mesial root of the first molar, appears to be determined at crown completion. Growth of the mandible in height appears to be completed at the cessation of root formation (approximately 26 days).

c. A downgrowth of the root into the jaw occurs at the initial stages of root formation or just after crown completion, and before eruption toward the occlusal begins.

d. Root completion and full eruption are closely related.

e. The greatest rate of growth occurs within the first 12 days.

f. Growth and development relationships cease to be correlated following root completion insofar as eruption of the tooth is concerned.

A MODIFIED GALLEGO'S IRON FUSCHIN STAIN AS A DIFFERENTIAL STAIN FOR CEMENTUM

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A SEARCH of the technical literature has shown that there is at present no technic which will differentially stain the cementum of the human teeth. This report concerns a successful differential staining of the human cementum. This stain is also successful for the cementum of rats' teeth and would presumably work on the cementum of all mammalian teeth.

A successful stain for cementum can be exceedingly useful in both investigation and teaching. For instance, it is of interest to be able to study the rate of cementum deposition in both humans and experimental animals and to study abnormal deposits of cementum. In the classroom it could be used advantageously in orienting beginning students in dental histology and in differentiating cemental portions of calcified odontomas.

TECHNICAL METHODS

The technic is derived from Lillie's modification of Gallego's iron fuschin stain. This technic may be found in Lillie (1948), German (1939), or Langeron (1934). The present procedure differs from those previously used in staining times and in the make-up of picro-anilin-blue.

Formalin fixation is recommended. The method of imbedding does not appear to be critical for the technic since equally good results have been obtained with decalcified paraffin, decalcified frozen and undecalcified ground sections. Minor variations in staining times are necessary, depending upon thickness of tissue and the type of calcification.

1. Sections are removed from 50 per cent alcohol and placed for 10 to 12 minutes in Weigert's acid iron chloride hematoxylin. (This is stored as 2 stock solutions, A and B. Solution A consists of 100 ml. of fresh 1 per cent hematoxylin in 95 per cent alcohol. Solution B consists of 95 ml. of distilled water, 1 ml. of concentrated hydrochloric acid, and 4 ml. of U.S.P. solution of iron chloride (37.2 Gm. of ferric chloride in 100 ml. of distilled water). Equal parts of stock solutions A and B should be freshly mixed together before use. Stock solutions last 2 to 3 weeks.)

2. Wash in distilled water.

3. Sensitize 30 seconds in a fresh mordant solution made by adding 200 ml. of distilled water to 1.5 ml. of concentrated nitric acid with 1 ml. of 40 per cent formaldehyde and 1.5 ml. of U.S.P. iron chloride added in the order given.

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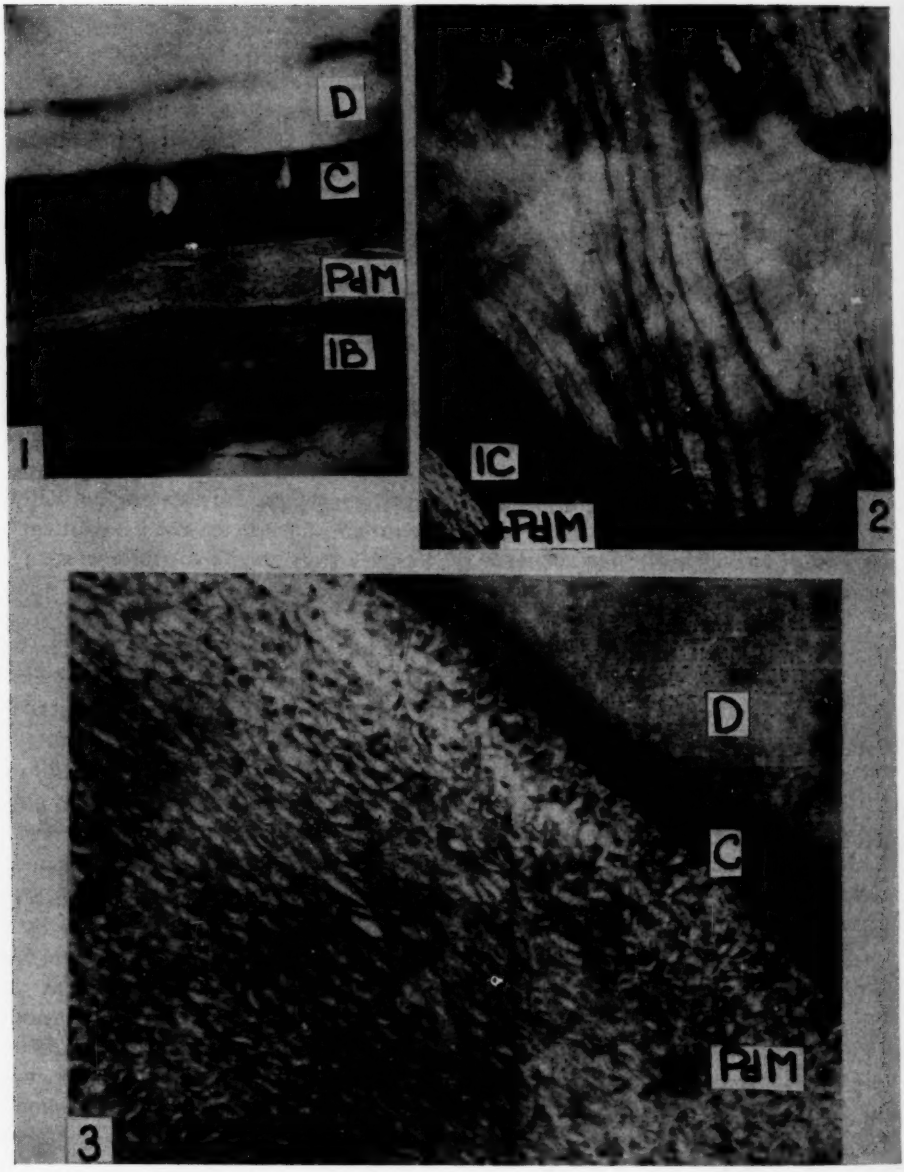

Fig. 1.—A decalcified frozen section of a mandibular incisor with extracted adherent bone. The deeply stained layer of cementum (*C*) contrasts markedly with the dentin (*D*) and periodontal membrane (*PdM*). The incremental lines of the alveolar bone (*IB*) stain in the same manner, but not so intensely as the cementum. (Orig. mag. ×200.)

Fig. 2.—A decalcified frozen section of a hypercementosis at the bifurcation of a mandibular molar. The incremental lines of cementum (*IC*) stain even more intensely than those of the alveolar bone. Periodontal membrane at the bifurcation (*PdM*). (Orig. mag. ×250.)

Fig. 3.—A paraffin section of a rat's incisor. Cementum (*C*) is strongly delineated from the dentin (*D*), periodontal membrane (*PdM*) and bone. (Orig. mag. ×250.)

4. Rinse in distilled water.

5. Stain 5 minutes in a fresh dilution of 3 ml. carbol fuschin in 50 ml. of 0.2 per cent acetic acid.

6. Rinse in distilled water.

7. Differentiate by returning to mordant for 1 to 2 minutes (depending on section).

8. Rinse in distilled water.

9. Stain 10 to 30 seconds in 0.01 per cent anilin blue in saturated picric acid solution.

10. Rinse in 0.1 per cent acetic acid.

11. Dehydrate and clear with xylene, and mount in balsam, (Frozen sections will shrivel up in xylene, so when clearing frozen sections use xylene, carbol-xylene sequence before mounting in balsam.)

RESULTS

Fig. 1, a decalcified frozen section of a mandibular incisor with adherent bone, shows the deeply stained layer of cementum (*C*), contrasting markedly with the dentin (*D*) and the periodontal membrane (*PdM*). The cementum is a deep red in contrast to the green stain of the dentin and bone.

It should be noted that the incremental lines of the alveolar bone (*IB*) stain somewhat less intensely, but the same color as the cementum. Indeed, in large cementoses, as can be seen in Fig. 2, a decalcified frozen section of a hypercementosis at the bifurcation of a mandibular molar, the incremental lines of cementum (*IC*) stain even more intensely than those of alveolar bone.

In Fig. 3, a paraffin section of a decalcified rat's incisor, the cementum (*C*) is clearly delineated from the dentin (*D*), periodontal membrane (*PdM*) and bone.

SUMMARY

A technic for relatively specific staining of human teeth is described. The method is empirical and certain of the reagents are irrational. No histochemical specificity should be imputed to the technic. Its research and teaching value lies in differentiating cementum from other hard structures.

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